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ENDOCRINOLOGY

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VOLUME 29 NOVEMBER, 1941 NUMBER 5

CALCIUM APPETITE OF PARATHYROIDECTOMIZED RATS USED TO BIOASSAY SUBSTANCES WHICH AFFECT BLOOD CALCIUM[1]

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BALTIMORE, MARYLAND

IT HAS LONG BEEN KNOWN that the removal of the parathyroids in experimental animals, as well as their accidental removal in man, is followed by a change in the electrolyte balance of the blood serum resulting in a lowering of the calcium-phosphorus ratio which may cause the characteristic signs of parathyroid deficiency, chief of which is tetany (1, 2, 3). In self-selection studies it was found that parathyroidectomized rats offered a calcium lactate solution in a container separate from the food and water drank sufficient calcium lactate solution to remain alive and free from signs of tetany (4). Postoperatively in several instances such rats increased their calcium lactate solution intake 10 to 15 times. Successful parathyroid implants to the anterior chamber of the eye decreased the calcium lactate intake to its normal level (5). The parathyroidectomized rats, having lost their internal or physiological means of maintaining a constant internal environment—in this case a normal calcium-phosphorus ratio—made an effort themselves to maintain the proper ratio by voluntarily ingesting larger amounts of calcium. The evidence at hand indicates that these rats drank more of the calcium lactate solution because of an altered taste for calcium and not because they learned that the ingestion of calcium brought relief from discomfort (6).

We were interested to know whether the calcium lactate appetite of parathyroidectomized rats is sufficiently sensitive to be used as a measure of the effects of various substances which affect calcium metabolism. For this purpose a study was made of the effects produced on the calcium appetite by increasing the calcium content of the food, by injecting parathyroid extract, or by feeding cod liver oil, dihydrotachysterol (A-T-10), vitamin D_2, irradiated ergosterol, vitamin D_3, or irradiated cholesterol. A comparison was made of the minimum effective doses of these different substances (the amounts necessary to return the calcium intake approximately to its normal level).

Received for publication August 2, 1941.

[1] This work was aided by a grant from the Committee on Research in Endocrinology of the National Research Council.

[2] Henry Strong Denison Scholar for 1941–42.

METHODS

The rats were kept separately in cages which contained a food cup and two graduated bottles, one filled with water, the other with a 2% calcium lactate solution. The food was made according to the following formula:

graham flour	725 gm.
casein	100 gm.
butter	50 gm.
salt	10 gm.

This is our regular stock diet with the skim milk powder (100 gm.) and calcium carbonate (15 gm.) omitted.

Daily records of the food, water, and calcium solution intake were taken. The rats were weighed weekly.

In order to obtain baselines, records were taken for two or more weeks before the parathyroids were removed. Therapy was not started postoperatively until the increased calcium lactate intake had reached a plateau, which usually required 30 to 40 days. In order to assure an even distribution of the vitamins in the food, they were dissolved in the food fat which was then mixed with the remaining food constituents.

The parathyroids were removed under a 15× magnifying binocular dissecting microscope by the aspiration method shown in figure 1. After exposure of the thyroid its capsule was torn near the parathyroid gland; with suction applied to a fine glass

Fig. 1. Drawing illustrating technic of parathyroidectomy in rat.

cannula the thyroid tissue surrounding the parathyroid was removed and the gland lifted free. Care was taken not to rupture the superior thyroid vessels; although in special cases in which they branched over or near the parathyroids they were ligated leaving only the inferior thyroid vessels to supply the thyroid gland. The constant aspiration kept the field clear and facilitated the removal of the parathyroids in their unruptured capsule.

The 31 rats used for these experiments varied in age from 60 to 180 days at the beginning of the experiment and included both males and females.

duced on the calcium lactate appetite by increasing the calcium content of the food. The amount of calcium lactate mixed with the food was somewhat greater than the

RESULTS

Calcium Lactate Added to the Food

In the first group of parathyroidectomized rats we determined the effects pro- rats had previously received from the calcium solution. Figure 2 shows the chart of a typical rat. It gives the daily intake of the calcium lactate solution (2%) and water and

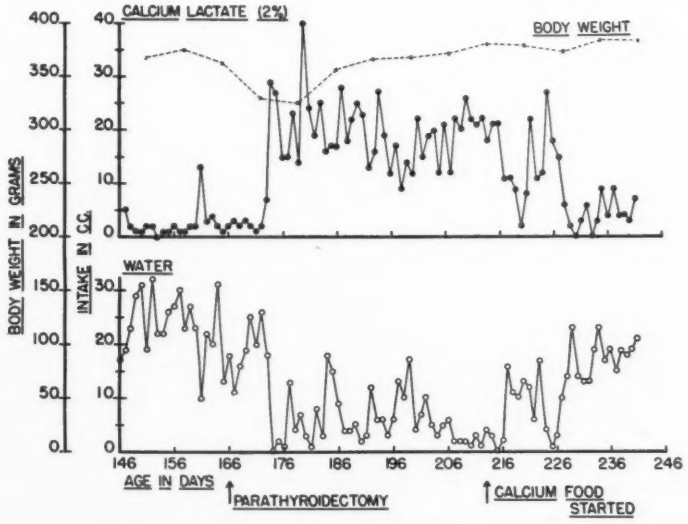

Fig. 2. Calcium and water intake in parathyroidectomized rats.

weekly body weight records. This rat was started at an age of 146 days, was parathy- roidectomized at 166 days, and was placed on the high calcium diet at 213 days. This animal increased the calcium solution intake from a daily average of 1.4 cc. for the 10 days preceding parathyroidectomy to 15.4 cc. for the last 10-day period postopera- tively. Calcium lactate was added to the food (73 gm. per 1000 gm. of food) on the 47th day postoperatively. Thirty days later the calcium lactate solution intake de- creased to an average of 2.9 cc. Water intake varied inversely with that of the calcium solution. Table 1 summarizes the results for the 5 rats. Calcium lactate solution intake increased from 2.6 cc. before to 21.2 cc. after operation and decreased to 5.7 cc. when the food contained calcium. Postoperatively the water intake decreased from 21.4 cc.

TABLE 1. CALCIUM AND WATER INTAKE OF PARATHYROIDECTOMIZED RATS ON A HIGH CALCIUM DIET
(7.3 gm. of calcium lactate per 100 gm. of calcium-free food)

Rat No.	Body Wt. in Gm.	Av. Daily Water Intake in cc.			Av. Daily Intake of Calcium Lactate Solution (2%) in cc.		
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy
1	211	18.5	4.3	13.6	1.4	15.4	2.9
2	410	22.9	6.2	13.7	1.4	17.3	6.1
3	240	18.7	1.4	24.2	7.3	33.5	6.3
4	266	22.6	3.0	17.5	1.9	19.5	4.5
5	355	24.5	6.4	19.0	1.2	20.5	8.5
Mean	296	21.4	4.3	17.6	2.6	21.2	5.7

to 4.3 cc. and increased to 17.6 cc. for the last 10 days of therapy (28 days total period). None of the rats developed tetany.

Parathyroid Extract

In another group of rats parathyroid extract[3] was administered. This group of 5 rats was given daily injections of the extract after the calcium ingestion had risen

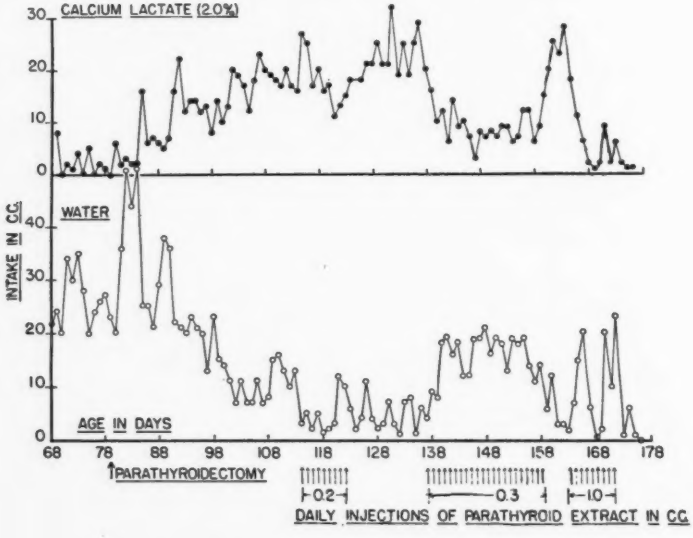

Fig. 3. CALCIUM AND WATER INTAKE IN PARATHYROIDECTOMIZED RATS RECEIVING PARATHYROID EXTRACT.

postoperatively to a fairly constant daily intake. Figure 3 shows the chart for one of these animals. This rat was placed in the experimental cage at an age of 68 days and was parathyroidectomized at 79 days. Calcium lactate intake before operation averaged 2.3 cc. per day and increased to an average of 18.0 cc. for the 10-day period pre-

[3] Parathormone, Eli Lilly and Company, Indianapolis, Indiana.

ceding the treatment with parathyroid extract. Daily injections of 0.2 cc. (20 u) given for 9 days produced only a slight transitory effect, if any at all. The dosage was then increased to 0.3 cc. after a 15-day interval during which no injections were given. Twenty-two daily injections resulted in a decrease of calcium lactate ingestion to a lower plateau (8.3 cc.) which was somewhat above normal. Water intake showed inverse changes; from a level of 24.8 cc. before operation it decreased to 4.2 cc. for the 10-day period preceding the 0.3 cc. injections and increased during therapy to 16.1 cc. During a 4-day interval between the last injection of 0.3 cc. and the increased dose of 1.0 cc., the calcium lactate intake rose rapidly to its previously high level. This increased dosage given for 9 days quickly reduced the calcium appetite to its normal level. However, the toxicity of the increased dosage made the rat extremely ill; it refused food, lost weight, and died 5 days after the last injection. This rat survived longer than most of the remaining 4, three of which died before injections were dis-

TABLE 2. CALCIUM AND WATER INTAKE IN PARATHYROIDECTOMIZED RATS RECEIVING PARATHORMONE THERAPY

(0.3 cc. or 30 u per day subcutaneously)

Rat No.	Body Wt. in Gm.	Av. Daily Water Intake in cc.			Av. Daily Intake of Calcium Lactate Solution (2%) in cc.		
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy
6	272	24.8	4.2	16.1	2.3	22.9	8.3
7	338	25.4	3.9	14.9	1.3	20.5	8.2
8	327	29.2	1.0	5.4	6.3	49.6	32.8
9	317	29.1	4.7	6.4	1.5	15.1	13.1
10	292	14.3	0.6	11.8	4.6	27.6	10.2
Mean	309	24.6	2.9	10.9	3.2	27.1	14.5

continued. Table 2 summarizes the results for the 30-day treatment period with 0.3 cc. per day. The calcium intake preoperatively averaged 3.2 cc., postoperatively 27.1 cc., and for the last 10 days of the injections 14.5 cc. Water intake averages for the same periods were 24.6 cc., 2.9 cc., and 10.9 cc. respectively. Thus, while this dosage of the hormone was effective, it was not a complete substitute as judged by the failure of the calcium intake to return to its normal level.

Dihydrotachysterol[4] (A-T-10)

The procedure used in determining the minimum effective dose of A-T-10 will be described in detail, since it was the same in all of the following experiments.

Having no idea of the dose of A-T-10 required to affect the calcium appetite, large doses (5 mg. per 100 gm. of food) were given in preliminary experiments. Figure 4 shows a typical record of one of the 3 rats. It gives the daily intake of calcium lactate solution (2%) and of water and the weekly record of body weight. The rat was put in the cage at an age of 120 days and was parathyroidectomized at 159 days. For the 10 days preceding parathyroidectomy the daily calcium lactate intake averaged 2.4cc. and the water intake 21.1 cc. After operation there was an immediate decrease in water intake and an increase in calcium lactate intake. After 40 days, when the calcium lactate intake averaged 16.4 cc. and the water intake 7.7 cc., 5 mg. of A-T-10 was added per 100 grams of food; and this diet was continued for 7 days. On the first day

[4] Winthrop Chemical Company, Inc., New York City.

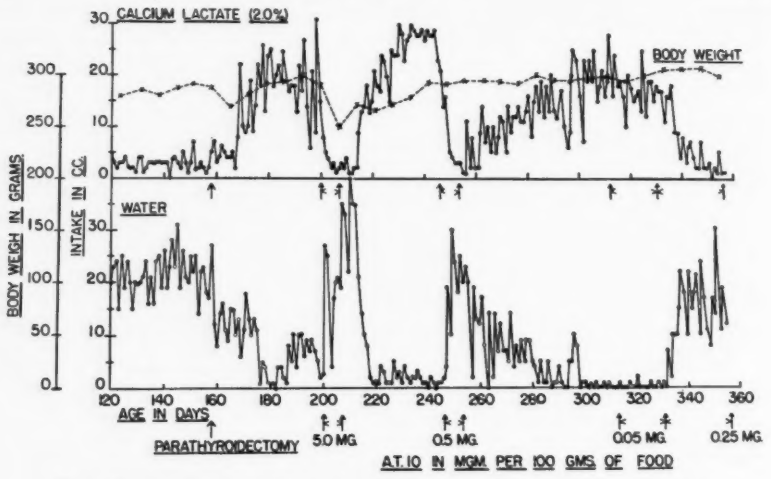

Fig. 4. Calcium and water intake in parathyroidectomized rats receiving A-T-10.

the water intake increased sharply from 3 cc. to 27 cc., the calcium solution intake decreased from 16 cc. to 8 cc. After 11 days the water had reached a peak of 40 cc. with a calcium intake of 1 cc. This dose was evidently toxic, as was indicated by a rapid loss of weight. About 10 days after the rats were returned to their special calcium-free diet, the calcium solution intake rose and the water intake decreased. In 20 days the calcium intake actually surpassed the level reached before A-T-10 was administered,

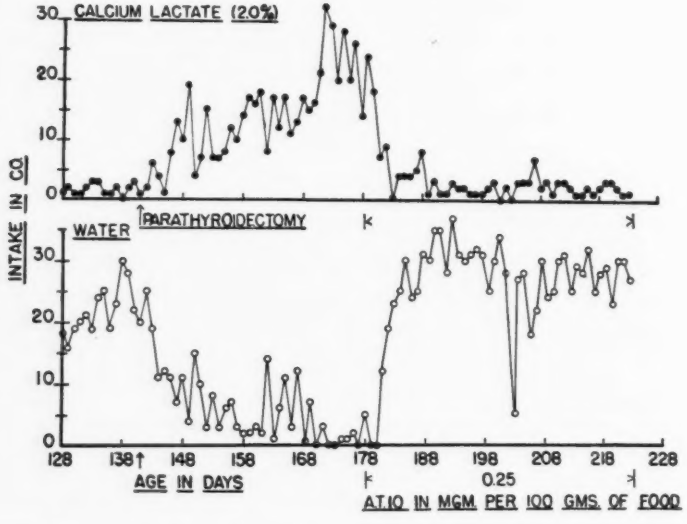

Fig. 5. Calcium and water intake in parathyroidectomized rats receiving A-T-10.

water intake being proportionately lower, thus keeping the total water nearly the same. After the intake of the fluids had become stabilized, 0.5 mg. of A-T-10 was mixed with each 100 gm. of food and continued for another 7 days. The results closely resembled those produced by the 10-fold stronger dose, except that body weight showed no change and the subsequent return to the original levels occurred less rapidly. Approximately 50 days later, when the intakes of calcium and water had again reached fairly constant levels, 0.05 mg. per 100 gm. of food was administered. Treatment with this dosage for 18 days had no noticeable effect. Increasing the dose to 0.25 mg. quickly caused the calcium lactate to return to its normal preoperative level. Thus, the minimum effective dose must lie between 0.25 and 0.05 mg. per 100 gm. of food. The other two rats gave almost identical records.

TABLE 3. EFFECT OF DIHYDROTACHYSTEROL (A-T-10) THERAPY ON CALCIUM AND WATER INTAKE OF PARATHYROIDECTOMIZED RATS

(0.25 mg. per 100 gm. of calcium-free food)

Rat No.	Body Wt. in Gm. at Beginning of Treatment	Av. Daily Water Intake in cc.			Av. Daily Calcium Lactate (2%) Intake in cc.			Av. Daily Food Intake in Gm.	Av. Daily Intake of A-T-10 in Mg.	Av. Daily Intake of A-T-10 in Mg./Kg. Body Wt.
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy			
11	296	20.1	4.1	16.2	4.0	15.8	2.4	9.7	0.0242	0.0818
12	304	21.7	0.3	21.2	2.4	19.8	2.6	12.5	0.0312	0.1026
13	283	22.2	1.8	15.5	1.3	17.0	2.2	11.7	0.0292	0.1031
14[1]	395	21.1	5.1	24.8	2.4	12.4	3.0	15.3	0.0766	0.1940
15	220	13.4	1.2	19.6	3.6	19.1	1.8	13.1	0.0328	0.1491
16	273	23.5	1.9	27.9	1.8	22.1	1.8	13.0	0.0325	0.1190
17	225	17.2	3.8	22.4	1.6	21.1	1.7	11.1	0.0278	0.1235
18	215	17.7	1.0	25.5	4.4	23.9	0.8	10.7	0.0268	0.1246
Mean	276	19.6	2.4	21.6	2.7	18.9	2.0	12.1	0.0351	0.1247

[1] A heavy rat responding to 0.5 mg. per 100 gm. of food only.

The actual amount of the minimal effective dosages of the A-T-10 per kg. of body weight was calculated from the daily food intake of these 3 rats and of 5 additional rats. Figure 5 gives a typical record of one of the latter rats which received a diet containing 0.25 mg. of A-T-10 per 100 gm. of food. Parathyroidectomy performed when this rat was 141 days of age increased the calcium lactate intake from 1.8 to 22.1 cc. and reduced the water intake from 23.5 to 1.9 cc. Treatment with A-T-10 started when the rat was 178 days of age, caused the calcium lactate intake to drop sharply from 22.1 cc. to an average of 1.8 cc. for the 10 days preceding discontinuation of therapy; however, as the figure shows, the intake of calcium only reached this low level 10 days after therapy was begun. Water intake varied inversely. Table 3 summarizes the results of the observations made on the 7 rats with 0.25 mg. dosage and the 1 rat receiving 0.5 mg. (a heavy rat which was responding too slowly). The average daily water intake decreased from 19.6 cc. for the 10-day period immediately preceding operation to 2.4 cc. for the 10 days preceding A-T-10 administration. Treatment increased the water intake to 21.6 cc., which is slightly higher than the preoperative level. The intake of the 2% calcium lactate solution increased for the same periods from a preoperative baseline of 2.7 cc. to a postoperative plateau of 18.9 cc. Therapy reduced this level to 2.0 cc. The average amount of A-T-10 received

daily, calculated from the food intake which averaged 12.1 gm., was 0.035 mg., or 0.125 mg. per kg. of body weight.

Vitamin D Preparations

Using a technic similar to the one outlined above, irradiated ergosterol,[5] crystalline vitamin D_2,[6] crystalline vitamin D_3,[6] and irradiated cholesterol[5] were added to the food of different groups of rats. In each instance we started with a high dosage of the

TABLE 4. EFFECT OF VITAMIN D_2 THERAPY ON CALCIUM AND WATER INTAKE OF PARATHYROIDECTOMIZED RATS
(0.23 mg. per 100 gm. of calcium-free food)

Rat No.	Body Wt. in Gm.	Av. Daily Water Intake in cc.			Av. Daily Intake of Calcium Lactate Solution (2%) in cc.			Av. Daily Food Intake in Gm.	Av. Daily Intake, D_2 in I.U.	Av. Daily Intake of D_2 in I.U./Kg. Body Wt.
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy			
23	406	28.7	11.1	16.4	5.0	23.9	15.8	17.7	1640	4040
24	404	41.1	16.5	38.6	1.8	20.6	3.1	16.4	1500	3720
25	392	24.1	20.1	34.7	1.2	11.5	3.0	15.7	1440	3680
26	404	19.2	7.6	15.3	3.4	15.0	8.8	15.3	1410	3480
Mean	402	28.3	13.8	26.3	2.9	17.8	7.7	16.3	1500	3730

TABLE 5. EFFECT OF IRRADIATED ERGOSTEROL THERAPY ON CALCIUM AND WATER INTAKE OF PARATHYROIDECTOMIZED RATS

Rat No.	Body Wt. in Gm.	Av. Daily Water Intake in cc.			Av. Daily Intake of Calcium Lactate Solution (2%) in cc.			Av. Daily Food Intake in Gm.	Av. Daily Vitamin Dosage in Mg./100 Gm. of Food	Av. Daily Intake of Vitamin, I.U.	Av. Daily Intake of Vitamin in I.U./Kg. Body Wt.
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy				
14	400	21.1	5.3	31.3	2.4	23.0	3.8	17.8	2.07	6100	15200
17	240	17.2	10.5	25.4	1.6	10.2	3.0	11.8	0.69	1340	5600
16	290	23.5	5.8	24.1	1.8	20.7	6.2	14.4	0.69	1650	5700
15	240	13.4	6.3	22.1	3.6	13.1	2.4	12.1	0.69	1390	5800
Mean	293	18.8	7.0	25.7	2.4	16.8	3.9	14.0	—	2620	8080

substance. Just as soon as the calcium lactate intake decreased to its originally low level, the dosage was discontinued and sufficient time was allowed to permit the calcium lactate intake to increase to its high level again. In successive trials the dosage was decreased by factors of 10 until the calcium lactate intake failed to decrease to almost its normal level. Then the dosage was increased by factors of 5, that is, to half of the present effective dosage. When this dosage decreased the calcium lactate intake to within 2.5 times its preoperative level, it was taken as the minimum effective dosage. Tables 4, 5, 6, and 7 show the vitamin intake of the rats in the different groups and averages of the intakes for the different vitamins on the last effective dose. The

[5] Alba Pharmaceutical Company, Inc., New York City.
[6] Squibb Institute for Medical Research, New Brunswick, N. J.

minimum effective dose in international vitamin D units per kg. of body weight for the various preparations was:

vitamin D_2 (crystalline)	3730 (4 rats)	table 4
irradiated ergosterol	8080 (4 rats)	table 5
vitamin D_3 (crystalline)	3380 (4 rats)	table 6
irradiated cholesterol	3770 (5 rats)	table 7

In the larger doses irradiated ergosterol and irradiated cholesterol produced the

TABLE 6. EFFECT OF VITAMIN D_3 THERAPY ON CALCIUM AND WATER INTAKE OF PARATHYROIDECTOMIZED RATS
(0.2 mg. per 100 gm. of calcium-free food)

Rat No.	Body Wt. in Gm.	Av. Daily Water Intake in cc.			Av. Daily Intake of Calcium Lactate Solution (2%) in cc.			Av. Daily Food Intake in Gm.	Av. Daily Intake of D_3 in I.U.	Av. Daily Intake of D_3 in I.U./Kg. Body Wt.
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy			
19	278	16.7	0.8	17.1	1.6	17.9	2.7	13.0	1030	3700
20	334	20.1	7.1	18.2	3.4	15.2	5.6	12.7	1000	3000
21	310	13.8	1.6	21.6	5.5	20.4	3.2	13.7	1100	3500
22	256	22.5	0.6	19.8	3.4	22.3	2.4	10.5	850	3300
Mean	295	18.3	2.5	19.2	3.5	19.0	3.5	12.5	1000	3380

TABLE 7. EFFECT OF IRRADIATED CHOLESTEROL THERAPY ON CALCIUM AND WATER INTAKE IN PARATHYROIDECTOMIZED RATS
(3.45 mg. per 100 gm. of calcium-free food)

Rat No.	Body Wt. in Gm.	Av. Daily Water Intake in cc.			Av. Daily Intake of Calcium Lactate Solution (2%) in cc.			Av. Daily Food Intake in Gm.	Av. Daily Intake of Vitamin in I.U.	Av. Daily Intake of Vitamin, I.U./Kg. Body Wt.
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy			
5	320	24.5	11.0	32.2	1.2	21.8	3.3	13.3	1220	3820
4	407	22.6	7.5	19.1	1.9	18.8	3.9	14.9	1370	3360
14	413	21.1	5.3	22.0	2.4	23.0	2.0	17.0	1560	3780
1	237	18.5	5.4	17.4	1.4	16.9	4.9	10.4	980	4140
23	431	28.7	4.9	22.8	5.0	21.2	9.5	17.7	1620	3770
Mean	362	23.1	6.8	22.7	2.4	20.3	4.7	14.7	1350	3770

same toxic effect as A-T-10, that is, a loss of weight and death. The remaining two vitamin D preparations were administered only in smaller doses which did not produce any toxicity.

Cod Liver Oil

Cod liver oil which has been so extensively used as an antirachitic substance because of its vitamin D content was also investigated. Increasing doses of cod liver oil were added to the food; these doses, varying from 0.6 cc. to 24.0 cc. of oil per 100 gm. of food, had little effect on the calcium appetite. The only change was a slight weight loss which was not permanent but was soon regained while therapy continued. Since there was no definite effect from feeding cod liver oil mixed with the food, 2.0 cc. of

cod liver oil per day were administered by stomach tube and continued for 18 days. The calcium appetite showed a small decrease, but this change could not be attributed to the effect of cod liver oil; for a slight decrease in weight was suggestive of other factors, such as toxicity. Another fact which supports this conclusion is the stationary water intake. An increase in water intake was usually the first sign of adequate vitamin D therapy. Table 8 gives the averages for the 5 rats on a daily dose of 2.0 cc. given by stomach tube. Water, which for a 10-day preoperative period averaged 22.3 cc., decreased to 5.4 cc. after operation and remained unchanged during therapy. Calcium increased from 3.7 cc. to a postoperative level of 17.3 cc. and declined to only 12.6 cc.

TABLE 8. EFFECT OF COD LIVER OIL THERAPY ON CALCIUM AND WATER INTAKE IN PARATHYROIDECTOMIZED RATS
(2 cc., or 161 U., of vitamin D per day by stomach tube)

Rat No.	Body Wt. in Gm.	Av. Daily Water Intake in cc.			Av. Daily Intake of Calcium Lactate Solution (2%) in cc.			Av. Daily Intake of C.L.O. in I.U./Kg. Body Wt.
		10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	10 Days before Operation	10 Days before Therapy	Last 10 Days of Therapy	
27	284	21.7	5.1	6.2	2.5	20.2	11.2	567
28	244	21.5	9.3	6.4	1.3	12.7	7.0	660
29	262	14.8	1.5	5.1	6.7	22.3	12.2	615
30	242	21.4	1.7	3.5	3.1	16.5	11.6	665
31	328	32.1	9.6	5.0	5.0	14.6	21.2	490
Mean	272	22.3	5.4	5.2	3.7	17.3	12.6	600

during treatment. In I.U. the average vitamin D intake was 600 per kg. of body weight per day. Since this figure is far below the 3000 to 8000 U found to be effective with the other vitamins, no change in calcium appetite could have been expected with cod liver oil of this potency.

DISCUSSION

The results of these experiments demonstrate the great sensitivity of the calcium appetite of parathyroidectomized rats to changes in calcium metabolism. Quantities as small as 0.03 mg. per day of vitamin D preparations and A-T-10 were sufficient to reduce the calcium appetite. This decrease in calcium appetite after drug administration frequently manifested itself within 24 hours, which may indicate that changes in the blood chemistry of rats are more rapid than those in humans, for MacBryde reports that the first changes in blood calcium in humans appear only after about 48 hours of A-T-10 therapy (7).

In making a comparison between amounts of vitamin D and A-T-10 which have been used in controlling hypoparathyroidism in patients and the amounts we found necessary to restore a normal calcium appetite in rats, it was found that per kilogram body weight doses agreed closely when the patients treated were those having a low (5 to 6 mg.) serum calcium indicative of destruction or absence of parathyroid tissue (2, 3). For rats the minimum effective dose of A-T-10 was 0.035 mg., or 0.125 mg. per kg. of body weight. Parathyroid deficient patients have been given doses which ranged from 1.6 to 10.0 mg., or 0.021 to 0.166 mg. per kg. of body weight (7, 9), assuming 60 kg. as average body weight. Vitamin D_2 has been used to treat hypocalcemic patients and has been found as effective as vitamin D_3, the dosage being from 3600 to 5000 U per kg. of body weight per day, although some patients were controlled with

830 U (8, 10). These levels approximate the 3730 U of D_2 per kg. we found beneficial for rats, D_3 likewise showing an equal efficacy (11). We found no report in the literature of the use of irradiated ergosterol and cholesterol as such in treatment of hypoparathyroidism.

In marked contrast, injections of parathyroid extract affected the calcium appetite only when given in extremely large doses. Rats required 1 cc., or 100 U, or an average of 320 U per kg. of body weight to reduce the calcium appetite. Parathyroid deficient patients have received doses of 100 to 500 U, or roughly 1.6 to 8.3 U per kg. of body weight (7, 9, 10); but the beneficial effects soon disappeared. Thus, the doses of extract required to reduce the calcium appetite of parathyroidectomized rats to its normal level far exceeded the doses used in the treatment of acute parathyroid deficiency in patients.

The greater potency of A-T-10 and of the vitamin D preparations as compared to parathyroid extract in affecting the calcium appetite may be explained by the fact that the A-T-10 and the vitamin D preparations act directly on the calcium metabolism, affecting both calcium absorption and phosphorus excretion, while the extract acts primarily on the phosphorus excretion, having no effect on calcium absorption (9).

These results indicate that the calcium appetite may serve as a means of bioassay of substances which affect calcium metabolism. The appetite responds to the most minute quantities of substances which affect calcium metabolism and often within only a few hours. A bioassay with this method should not require very much time. Noteworthy is the fact that adult as well as young animals can be used with this method.

At present it is not possible to give a definite answer to the question of the mechanism underlying the changes in appetite. The evidence at hand indicates that these selections do not depend on a trial and error process (6). It seems more likely that they depend primarily on taste. Thus, the organism deprived of its parathyroid glands had a low blood calcium; and the changes in blood calcium affect all of the organs of the body, including the taste buds of the tongue. The presence of a high or low amount of calcium in the blood must thus affect the taste for calcium.

These changes in calcium appetite must be considered part of a mechanism by means of which the total organism helps to maintain a constant internal environment. In the intact animal this total organism regulation is not apparent, since much of the regulation is taken care of by physiological mechanisms. However, after removal of the physiological regulation, as in the present instance after removal of the parathyroid glands, the regulation of the total organism becomes evident. In the intact animal the physiological and total organism mechanisms undoubtedly must constantly supplement one another in maintaining a constant internal environment.

SUMMARY

Thirty-one parathyroidectomized rats kept on a low calcium diet but given access to calcium solution showed a greatly increased calcium appetite. By virtue of this increased calcium intake the rats survived and showed no tetany. Apparently they made an effort to maintain the blood calcium at its normal level.

In 5 parathyroidectomized rats increasing the calcium content of the food decreased the calcium appetite to approximately the normal level.

Parathyroid extract injected daily into 5 parathyroidectomized rats did not reduce the calcium appetite to its normal level until doses of 100 U per day were administered. This dosage was definitely toxic.

Dihydrotachysterol (A-T-10) given to 8 parathyroidectomized rats in the food in doses of as little as 0.035 mg. per day reduced their calcium appetite to its normal level.

Irradiated ergosterol and cholesterol and crystalline vitamins D_2 and D_3, given to 4 groups of 4 or 5 parathyroidectomized rats each respectively in minute amounts, 3000 to 8000 I.U. per kg. of body weight, sufficed to reduce the calcium lactate intake to its normal level.

Cod liver oil given to 5 parathyroidectomized rats either in the food or by stomach tube could not be given in sufficiently large amounts to reduce the calcium appetite to its normal level.

A comparison with the dosage used clinically for parathyroid deficient patients showed that when measured in terms of per kilogram body weight the effective doses of A-T-10 and of the vitamin D preparations given to the rats were about the same as have been used for the patients. The doses of parathyroid extract were far greater. The more direct action of the A-T-10 and of the vitamin D preparations on the calcium metabolism may explain the difference of effects.

The appetite method may be used for the bioassay of substances which affect the calcium metabolism.

We are indebted to the following pharmaceutical houses: Winthrop Chemical Company, Inc., Alba Pharmaceutical Company, Inc., the Squibb Institute for Medical Research, and Eli Lilly and Company.

REFERENCES

1. MacCallum, W. G., and C. Voegtlin: *J. Exp. Med.* 11: 118. 1909.
2. Thomson, D. L., and J. B. Collip: *Physiol. Rev.* 12: 309. 1932.
3. Dragstedt, L. R.: *Physiol. Rev.* 7: 499. 1927.
4. Richter, C. P., and J. F. Eckert: *Endocrinology* 21: 50. 1937.
5. Richter, C. P. and J. F. Eckert: *Amer. J. Med. Sc.* 198: 9. 1932.
6. Richter, C. P.: *Endocrinology* 24: 367. 1939.
7. MacBryde, C. M.: *J.A.M.A.* 111: 304. 1938.
8. Klatskin, G.: *J. Clin. Invest.* 17: 431. 1938.
9. Albright, F., H. W. Sulkowitch and E. Bloomberg: *J. Clin. Invest.* 18: 165. 1939.
10. Poer, D. H.: *South Med. J.* 33: 1174. 1940.
11. Himsworth, H. P., and M. Maizels: *Lancet* 1: 959. 1940.

Addendum

Since this paper was submitted for publication, F. C. McLean (J. A. M. A., vol. 117, p. 609, 1941.) in a review of parathyroid deficiency and its treatment with activated sterols maintains that there is essentially little difference in vitamin D preparations and A-T-10 in their efficacy in producing and maintaining normal calcium serum levels in this deficiency. This paper contains evidence and support for his point of view. When a weight comparison is made between vitamin D_2, vitamin D_3, and A-T-10, there seems little reason to select one rather than the other in the treatment of this glandular deficiency.

GROWTH RESPONSE OF THYROIDECTOMIZED GOATS TO ARTIFICIALLY FORMED THYROPROTEIN[1]

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THE EFFECT OF THYROXINE OR THYROID SUBSTANCE in stimulating increased milk and butterfat production in lactating dairy cattle has been amply demonstrated (1–4). The reports of Winchester (5) and Taylor and Burmester (6) indicate that thyroid active substances are also essential for egg production. Though present results are contradictory (7), there is a definite possibility that the growth processes of domestic animals may be stimulated by administration of suitable amounts of thyroid hormone.

Since the excessive cost of thyroxine and desiccated thyroid makes their use in animal production impracticable, the attention of the authors has been directed to a search for less expensive sources of thyroid active substances.

As early as 1914 Morse (8), confirmed by Rogoff and Marine (9) reported that iodized proteins produced slight physiological effects, qualitatively comparable to that produced by thyroid iodine. More recently, Abelin and Florin (10), and Lerman and Salter (11) have reported that the iodination of proteins, followed by hydrolysis, and concentration of the active material produced substances exerting high thyroid activity. Ludwig and Mutzenbecher (12) reported the actual isolation of crystalline thyroxine from such preparations.

With these findings in mind experiments were undertaken to devise a method of forming physiologically active thyroproteins *in vitro* with sufficient potency to be effective when given orally without preliminary hydrolysis and concentration of the active principle. In a preliminary report one of the authors (13) presented evidence of the lactation-stimulating properties of artificial thyroproteins when fed to lactating goats. At this time, the name 'thyrolactin' was proposed for this preparation. The present paper is concerned with further evidence of the physiological potency of such preparations. Details of production and assay will be presented in a later communication.

During the routine testing of numerous preparations of iodinated casein and other milk proteins extensive data have accumulated indicating the efficacy of these preparations in hastening the metamorphosis of tadpoles and increasing the metabolic rate of guinea pigs. Since phenol derivatives other than thyroxine are known to produce qualitatively similar effects, it was believed that a more crucial test would be provided by determining the growth-stimulating properties of our materials in thyroidectomized animals.

That the thyroid gland is an important factor in the stimulation of growth and the

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metabolic processes associated with it is shown by the well established fact that when the thyroid is removed from young animals there is an early cessation of growth and the development of typical symptoms of cretinism.

Simpson (14, 15) reported the effects of thyroidectomy and thyroparathyroidectomy in sheep and goats. Removal of the thyroids with contained internal parathyroids had little effect in adult sheep and in lambs 7 to 8 months old. However when the operation was performed 3 weeks to 2 months after birth typical cretinism together with marked stunting of growth resulted. Similar results were obtained with 2 female goats thyroidectomized at 20 days of age.

The investigations of Salmon (16), Evans, Simpson and Pencharz (17) and others have demonstrated that the effects of thyroidectomy can be overcome readily by the administration of thyroxine or thyroid substance. Evans, et al. reported that the injection in suitable amounts of either thyroxine or anterior pituitary growth hormone resulted in normal growth of thyroidectomized rats, while injection of both materials together produced growth above normal. In thyroidectomized-hypophysectomized rats, injected growth hormone caused normal growth, whereas thyroxine alone was without effect.

Thyroidectomized male goats have been used in this laboratory since 1938 in further studies of the thyroid-pituitary relationship as related to growth, the results of which have been published (20). Certain of the growth data following thyroidectomy obtained in the course of this study are included in this paper.

EXPERIMENTAL

Operative technic. So far as the authors are aware, the technic of thyroidectomy in the goat has not been described elsewhere, and therefore a brief description is given here. The thyroid of the goat is a bilobed gland, located a short distance behind the larynx, and with the right and left lobes, respectively, lying lateral to the trachea. The 2 lobes are connected by a thin isthmus of thyroid tissue arising medially from the caudal portion of each lobe, and passing across the ventral aspect of the trachea. In very young animals the 2 lobes of the thyroid are often embedded in thymus tissue. The chief blood supply is provided by the thyroid artery, entering medially at the cephalic end, and the thyroid vein located medially in the central portion of each lobe.

Preliminary to thyroidectomy the goat is placed on its back in a v-shaped operating table and its legs are securely tied. The ventral portion of the neck is shaved, swabbed with 70% ethyl alcohol and painted with tincture of iodine. The thyroid region is anesthesized by subcutaneously injecting 2 cc. of a 2% apothesine solution, thoroughly infiltrating it through the operative region. A midline incision about 2 inches long is made caudal to the larynx, and the skin is freed from underlying structures by blunt dissection, the operative field being protected by clipping sterile towels to tissues underlying the incised edges of the skin. The sterno-hyoideus muscle is retracted medially and the sterno-cephalicus muscle is retracted laterally, exposing the right lobe of the thyroid lying beneath it. By passing a blunt probe beneath the lobe of the gland it can be raised sufficiently to permit clearing it of surrounding connective tissue. This exposes the thyroid vein and artery which are then drawn together by passing a silk ligature beneath them, and both are tied off together. The vessels are cut between the ligature and the gland. The right lobe is left in place attached to the isthmus, and the left lobe is then freed and the vessels are tied off in a similar manner. The right lobe is then passed beneath the sterno-hyoideus muscle permitting both lobes to swing free still connected by the isthmus. The isthmus is freed by care-

ful dissection from the connective tissue surrounding it and the entire thyroid removed in toto. The operative incision is then closed either with Michel wound clips or linen sutures and the incision is painted lightly with tincture of iodine.

Young goats show very little immediate effects of the operation. They usually eat normally within an hour after being released, and show no noticeable effects the next day. If thyroidectomized within the first week of life visible signs of cretinism and thyroid deficiency appear within the first 2 weeks following the operation. If thyroidectomized later it takes somewhat longer for the symptoms to develop.

The goat is a favorable subject for thyroidectomy in that the 2 external parathyroids are located at the head of the thymus some distance from the thyroid gland. When the thyroid is removed together with the 2 parathyroids contained within it, the external parathyroids continue to function, with the result that complications due to parathyroid deficiency are not encountered.

Description of animals. The goats used in these experiments were grade Toggenburgs. The kids were weaned at 2 or 3 days of age and received goats' milk, supplemented by cows' skimmilk when necessary until about 3 months old. In addition good quality alfalfa hay and a concentrate mixture were provided ad libitum.

Ten male goats were thyroidectomized between the ages of 5 and 24 days and allowed to develop with no replacement therapy. Preliminary work (unpublished) had disclosed that thyroidectomized animals are extremely susceptible to respiratory infections and digestive disturbances, and that when operated upon at an early age there is a heavy mortality after the fourth month. Since measurements of the internal glands and organs were desired for a related problem, these animals were killed at about 4 months of age. Post mortem examination revealed small fragments of thyroid remaining in 2 animals and therefore only 8 of this group are included in the present report. Because it was impracticable under our conditions to retain normal males for controls, growth data were obtained on 4 normal females for comparison with the thyroidectomized animals.

Six additional animals, 4 males and 2 females, were thyroidectomized on the same day, and were given no further treatment until visible symptoms of thyroid deficiency developed. At the end of the 4th postoperative week feeding of thyrolactin in graded amounts was begun. All animals were weighed at weekly intervals.

Effects of thyroidectomy. Symptoms of thyroid deficiency developed rapidly in young animals. By the end of the second week the kids acquired a dull, sleepy appearance, usually stood in a relaxed position, and had a diminished appetite. There was a rapid decline in the growth rate and there was complete growth stasis in most animals by the end of the second month. Coincident with this there was a shortening and thickening of the body and an enlargement of the paunch. Growth of the muzzle and jaw bones lagged behind that of the upper portions of the head with the result that an extremely dishfaced appearance developed.

Typical symptoms of cretinism are shown by the 3 thyroidectomized males pictured in figure 1. For comparison a normal female 30 days younger than the operated animals was included. The average growth curve of 8 thyroidectomized males is shown in comparison with a curve for normal females in chart 4, figure 2. It should be borne in mind that the growth rate for females is considerably less than for male goats and therefore the growth retardation due to thyroidectomy is actually greater than indicated by the chart. However, it is clearly demonstrated that total thyroidectomy of young goats produces a complete growth stasis by the first to second month following the operation.

The 6 kids receiving thyrolactin as replacement for the thyroid hormone were

divided into 3 groups and treated as follows: The two females, *kid* 97 and 100 received 0.3 gm. daily; *kid* 95 and 96 received 0.5 gm.; and *kid* 75 and 94 received 1 gm. daily. In order to insure consumption of the proper amounts, this material was given individually in gelatine capsules.

The results of the treatment are illustrated by the individual growth curves depicted in figure 2, charts 1, 2, and 3. It will be noted that by the fourth week after thyroidectomy there was a downward dip in the growth curve of each of the animals,

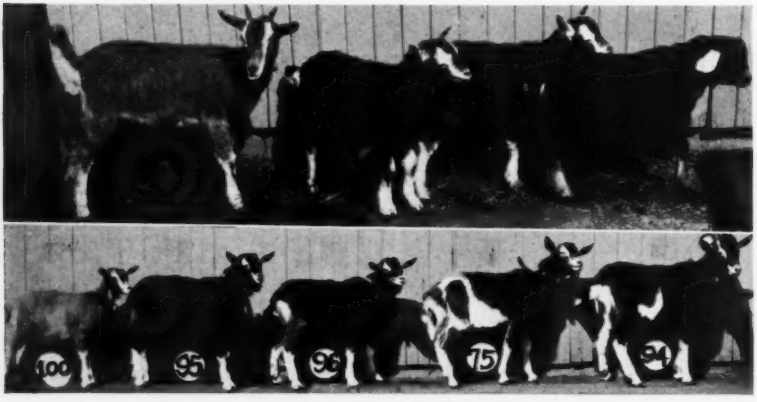

Fig. 1. THE EFFECT OF THYROIDECTOMY AND THYROPROTEIN FEEDING ON THE GROWTH AND BODY CONFORMATION OF YOUNG GOATS. *Upper photo.* SYMPTOMS OF THYROID DEFICIENCY. The 3 male kids at the right were thyroidectomized at 2 weeks of age and were 4 months old when photographed. They show marked growth retardation and advanced cretinism. The animal at the left is a normal female 30 days younger. *Lower photo.* GROWTH STIMULATION FOLLOWING THYROPROTEIN FEEDING. Beginning 1 month after thyroidectomy, thyrolactin was fed daily to these animals as follows: Kid 94 and 75, 1 gm.; Kid 95 and 96, 0.5 gm.; Kid 100, 0.3 gm. The growth obtained is roughly proportional to dosage. Body conformation is normal.

indicating the development of thyroid deficiency. In addition all of these animals had physical symptoms of early cretinism.

Within a week after the thyrolactin feeding was begun the growth curves resumed their upward trend and growth continued more or less uniformly for the duration of the experiment. The amount of growth obtained at the different feeding levels was roughly proportional to the amount of thyrolactin supplied.

The 2 animals receiving 0.3 gm. daily showed very little growth, their curves running parallel until reaching the age of 16 weeks. At this point *kid* 97 showed a rapid increase in growth which for some time remained a mystery. Toward the end of the experiment it happened that this animal developed an impacted rumen and was killed because there seemed no hope for her recovery. Post mortem examination revealed that she was pregnant with an approximately 4-month-old fetus. Calculating back, it was estimated that this animal had been bred at about 3 months of age. Since these kids were all confined in the same pen it was certain that one of the thyroidectomized males was the sire of the fetus. The weight of the pregnant uterus and its contents was only 6 pounds; yet the growth of *kid* 97 was more than double that of *kid* 100 on the same plan of treatment. It thus appears that the hormone lack of the mother was remedied either by diffusion of thyroid hormone from the fetus into the maternal circulation or by some other undefined factor related to the pregnant state.

Even though the dosage of thyrolactin alone was insufficient to support growth in these 2 animals it arrested the symptoms of cretinism and maintained a normal body conformation. *Kid* 100, uncomplicated by pregnancy, appeared normal in conformation though undersized at the age of 8 months.

Male *kid* 95 and 96 receiving 0.5 gm. of thyrolactin daily showed an accelerated growth rate as compared to the animals on the lower dosage but somewhat less growth than the remaining 2 that received 1 gm. daily. However, the last-named kids were older and somewhat heavier at the time of thyroidectomy, and this may account to some extent for the difference in growth. Normal male and female controls were not

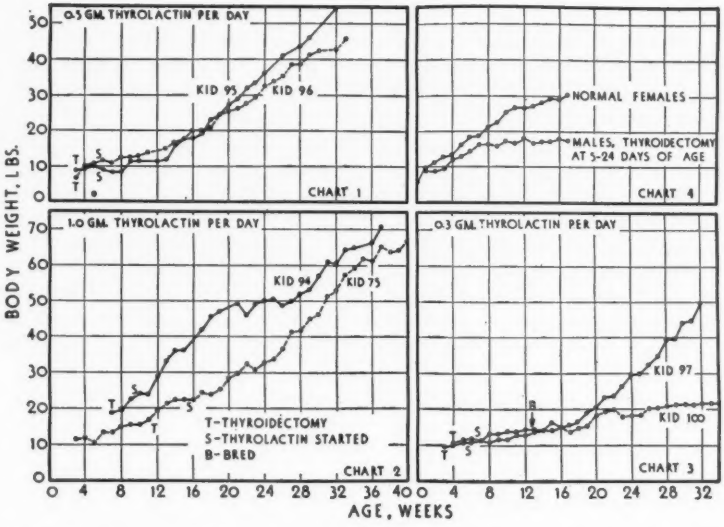

Fig. 2. The effect of thyroidectomy and thyroprotein feeding on the growth rate of young goats. *Charts* 1, 2 and 3 show the effect of feeding thyrolactin to thyroidectomized kids in graded dosages. *Chart* 4 illustrates the growth rate of thyroidectomized males as compared to normal females.

maintained for a sufficient length of time for comparison with animals in this experiment. Brody (18) has published growth data for young goats reared under very similar conditions of feeding and management. According to these data the normal weight at 32 weeks of age is 48 lbs. for females and 72 lbs. for males. When computed at the same age, *kid* 100 (0.3 gm. thyrolactin daily) is only 46% of the normal weight; *kid* 95 and 96 are 76 and 60% and *kid* 75 and 94 are 74 and 84% of normal, respectively.

A photograph of 5 of the thyroidectomized kids after more than 7 months maintenance by the feeding of thyrolactin is shown in figure 1. It will be noted that the appearance of these animals is normal except for somewhat retarded growth on the lower dosage levels. The growth of animals on the higher dosage levels approached, but did not attain the normal figure. It is believed, however, that the early retardation of growth following thyroidectomy, before replacement therapy was inaugurated, would cause the growth to lag somewhat behind the normal, even though optimum conditions were supplied during the course of treatment. Secondly, it is unlikely that the exact hormone requirement would be supplied by the arbitrary dosages given. When these limitations are considered, it appears that the artificial thyroprotein

would support normal growth in thyroidectomized goats if the treatment were begun immediately after operation and dosages were adjusted exactly to the requirements of the individual animals.

After about 8 months on the thyrolactin treatment the 4 males were killed, the remaining female being retained for further experimentation. Careful dissection of the thyroid region failed to disclose remnants of thyroid tissue in any of the animals. All the viscera and internal organs appeared entirely normal upon gross examination. No evidence was observed of toxic or irritating effects due to the relatively large amounts of organically combined iodine ingested.

DISCUSSION

In confirmation of earlier work, the experiments reported conclusively show that when young animals are thyroidectomized soon after birth a growth plateau is reached in a short time. The growth stasis is accompanied by development of marked symptoms of cretinism.

Experiments with small animals (16, 17, 19) have established the fact that these deficiency symptoms can be corrected by administration of suitable amounts of thyroxine or thyroid substance. Lerman and Salter (11) reported the correction of myxedema in adult humans by administration of a concentrate of the acid insoluble peptone obtained after the enzymatic hydrolysis of iodinated serum proteins.

The present work demonstrates that a thyroprotein prepared by the iodination of skimmilk proteins and administered orally without a preliminary hydrolysis arrests the development of cretinism and supports growth in thyroidectomized animals. Thus it would appear to have a physiologic action fully comparable to that of thyroid substance itself.

In view of the importance of growth processes in all phases of animal production the physiological mechanism whereby thyroid-active substances exert their effect is of considerable interest. Growth is generally conceded to be governed by a hormone or hormones of the anterior lobe of the pituitary since hypophysectomized animals fail to grow, but attain normal growth following the administration of pituitary substance. Recent work in our laboratory (20, 21) has led to the theory that the thyroid affects growth indirectly by stimulating the pituitary gland to greater production of certain hormones of the growth and metabolism complex. If this view is correct, it points to the possibility of increasing the rate of growth in certain instances by the administration of thyroproteins. In meat animals such as hogs and beef cattle the need for strains that will fatten readily at a desirable market weight has resulted in the development of animals with a relatively inactive thyroid, this resulting in turn in a slower rate of growth during early life. Rapidly growing strains of hogs have a higher pituitary thyrotropic hormone content than animals of a slow growing strain (22). It appears highly probable that the feeding of thyroprotein at the proper level to such slow growing animals would result in a more rapid rate of growth. When the desired skeletal size is attained such animals should fatten readily upon withdrawing the thyroprotein from the ration. In fact, it has been shown by Andrews and Bullard (23) that partial thyroidectomy of rapidly growing steers that failed to deposit body fat was followed by increased fat deposition and greater gains in body weight. The possibility of using thyroproteins to stimulate increased lactation has been indicated previously (13). Further research on this phase of the problem is now in progress.

SUMMARY AND CONCLUSIONS

The effect of total thyroidectomy on the growth and body conformation of young

male goats together with evidence of alleviation of the symptoms by the oral administration of an artificial thyroprotein in small amounts is reported.

When thyroidectomized during the first month of life young goats reach a complete growth stasis during the first 1 to 2 months after the operation. The growth failure is accompanied by typical symptoms of cretinism.

The oral administration of an artificial thyroprotein, produced by the iodination of skimmilk proteins arrests the development of cretinism and stimulates growth approaching that of the normal. Within the range of dosage covered the growth is roughly proportional to the amount of thyroprotein given.

From the results obtained it is concluded that the thyroprotein used has a physiologic activity fully comparable to thyroid substance.

The possible utilization of thyroprotein in animal production practices is discussed.

The technic of thyroidectomy in the goat is described.

REFERENCES

1. Graham, W. R., Jr.: *J. Nutrition* 7: 407. 1934.
2. Graham, W. R., Jr.: *Biochem. J.* 38: 1368. 1934.
3. Herman, H. A., W. R. Graham, Jr. and C. W. Turner: *Mo. Agr. Exp. Sta. Res. Bul.* 275. 1938.
4. Ralston, N. P., W. C. Cowsert, A. C. Ragsdale, H. A. Herman and C. W. Turner: *Mo. Agr. Exp. Sta. Res. Bul.* 317. 1940.
5. Winchester, C. F.: *Mo. Agr. Exp. Sta. Res. Bul.* 315. 1940.
6. Taylor, L. W., and B. R. Burmester: *Poultry Sci.* 19: 326. 1940.
7. Schneider, B. A.: *Quart. Rev. Biology* 14: 289 and 431. 1939.
8. Morse, M.: *J. Biol. Chem.* 19: 421. 1914.
9. Rogoff, J. M., and D. Marine: *J. Pharmacol. and Exp. Therap.* 19: 321. 1917.
10. Abelin, I., and A. Florin: *Arch. f. exper. Path. u. Pharmakol.* 171: 443. 1933.
11. Lerman, J., and W. Salter: *Endocrinology* 25: 712. 1939.
12. Ludwig, W., and P. von Mutzenbecher: *Ztschr. f. physiol. Chem.* 258: 195. 1939.
13. Turner, C. W.: *J. Dairy Sci.* 23: 535. 1940.
14. Simpson, S.: *Quart. J. Exp. Physiol.* 6: 119. 1913.
15. Simpson, S.: *Quart. J. Exp. Physiol.* 14: 161. 1924.
16. Salmon, T. N.: *Endocrinology* 23: 446. 1938.
17. Evans, H. M., M. Simpson and R. I. Pencharz: *Endocrinology* 25: 175. 1939.
18. Brody, S.: *Mo. Agr. Exp. Sta. Res. Bul.* 291. 1938.
19. Basinger, H. R.: *Arch. Int. Med.* 17: 260. 1916.
20. Reineke, E. P., A. J. Bergman and C. W. Turner: *Endocrinology* 29: 306. 1941.
21. Bergman, A. J., and C. W. Turner: *Endocrinology* 29: 313. 1941.
22. Elijah, H. D.: Masters' thesis. Univ. of Mo. 1940.
23. Andrews, F. N., and J. F. Bullard: *Proc. Am. Soc. Animal Prod.* 112. 1940.

EFFECT OF THYROID AND THYROTROPIC HORMONES UPON OXYGEN CONSUMPTION (QO_2) OF THE THYROID OF THE GUINEA PIG

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THE EXPERIMENTS reported in this paper were undertaken to throw light upon the interrelationship between the thyroid and the anterior pituitary glands. Evaluation of the activities of the thyroid and thyrotropic hormones was made upon the basis of observations of the effect of these hormones upon the oxygen consumption of guinea pig thyroid *in vitro*.

METHOD

The oxygen consumption ($\mathcal{Q}O_2$) of guinea pig thyroid tissue, when immersed in a medium containing the material to be tested, was measured by means of the Warburg apparatus. Standard technic for this apparatus was employed, that is to say, one hour at 38°C. with 100 agitations per minute. Thyroid glands of guinea pigs were sliced with very fine scissors and distributed among the vessels of a series of manometers in such a fashion that each vessel contained the same number of slices from each individual thyroid gland. The total quantity of thyroid in each vessel was equivalent to approximately 10 to 15 mg. of dried tissue. In each Warburg vessel there would be slices from each of six pigs. In this way the attempt was made to secure the greatest possible uniformity of thyroid tissue in the several vessels.

The medium used for the immersion of thyroids was the serum of normal humans. The action of such sera without the addition or extra hormones was first studied. In one observation the sera of *subjects A, B,* and *C* gave the following results for $\mathcal{Q}O_2$: 4.38, 4.56 and 4.42, respectively. In another observation with thyroids from a second lot of pigs the following were obtained: $\mathcal{Q}O_2$ 3.93, 3.81 and 3.88 for *A, B,* and *C,* respectively. The differences in each series are within the limits of acceptable error for this technic. From this it was concluded that the sera of these 3 normal subjects constituted a sufficiently constant medium for the purposes of the experiments.

The pH of sera was brought to 7.3 to 7.4 by the following procedure. HCl was added until pH 6.0 was reached, then the serum was shaken in the air, and thereafter placed in a vacuum for 2 hours. Following this it was placed in the refrigerator for 12 hours. At the moment of using the serum, its pH was raised to the physiological level by NaOH.

Thyroid hormone and/or thyrotropic hormone were added to sera so treated in determined amounts. Thyroid was given in the form of thyroglobulin made from human thyroid glands[2] having an iodine content of 36γ per cc. It was got into solution at a pH of 8.0. Several dilutions were then made with Ringer's solution, and the pH

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[1] Research Fellow, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts. Formerly from Ramos Mejia Hospital, Buenos Aires.

[2] For this material the author is indebted to Dr. Jacob Lerman.

brought to the physiological level by means of NaOH. The quantity of thyroid hormone added to sera was measured in terms of its iodine content. It was in the neighborhood of 15 to 120 γ% of the mixture.

Thyrotropic hormone was added in the form of Antuitrin T.[3] The quantity added was expressed in terms of the biologic unit used by the manufacturer.[4] The amounts used were between 10 and 30 U.

EXPERIMENTAL RESULTS

The experiments performed can be divided into three groups: those in which thyroid hormone alone was added; those in which thyrotropic hormone alone was added;

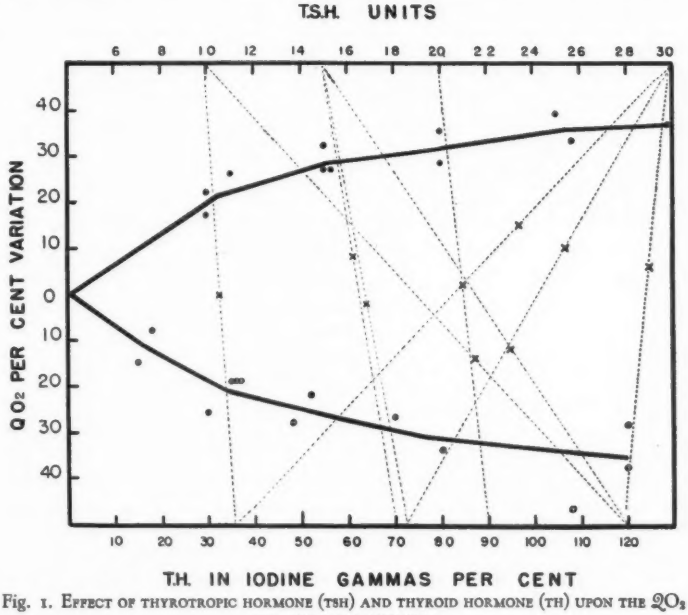

Fig. 1. Effect of thyrotropic hormone (TSH) and thyroid hormone (TH) upon the QO_2 of guinea pig thyroid.

and those in which both hormones were added. With thyroglobulin alone, 13 observations were made; with thyrotropin, 11 observations; and with both hormones, nine observations. The values for QO_2 obtained were referred to that of control serum (i.e., without added hormone) and were expressed as a percentage variation therefrom.

The results are shown in figure 1. In this graph the ordinate represents the percentage variation of QO_2 observed, from control serum. The lower abscissa represents the quantity of thyroglobulin in gammas of iodine per cent. The upper abscissa represents the quantity of thyrotropic hormone in biological units.

Through the 11 points obtained with increasing quantities of thyrotropic hormone, represented by solid dots, the most probable curve was drawn; and the same for

[3] The author wishes to thank Parke, Davis and Company for the generous gift of a supply of this material.

[4] It was found that considerable variation existed in the calorigenic action of different lots of Antuitrin T. The experiments reported here, however, all were done with one lot (#097758A); therefore any error due to variation in the strength of lots was avoided.

the 13 points obtained with thyroglobulin, shown by circles. It is evident that these two curves are approximately reciprocal.

The points for experiments in which both hormones were used were plotted as crosses. A line was drawn from the point in the top abscissa, denoting the quantity of thyrotropic hormone, to that on the lower, denoting thyroglobulin, and the point placed where the appropriate ordinate intersected this line. It can be seen that the actual result depends upon the additive effects of the actions (one stimulating, that is to say positive; the other depressing, that is to say negative) of the two hormones

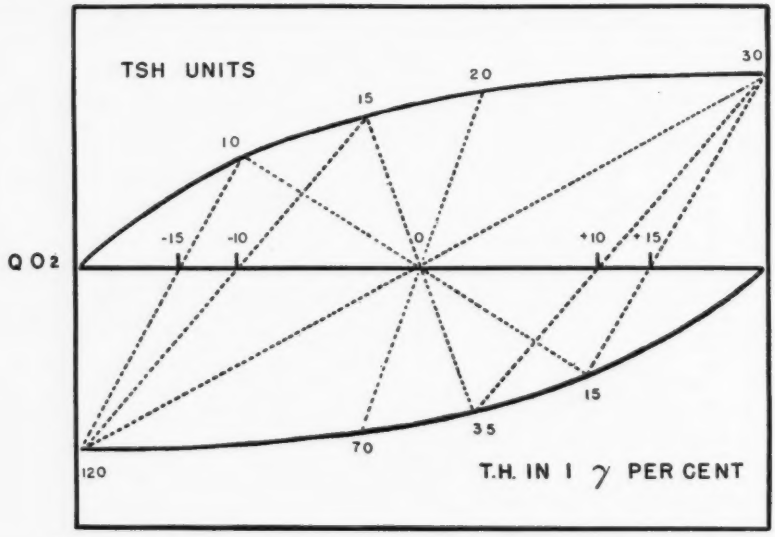

Fig. 2. Effect of thyrotropic hormone (TSH) and thyroid hormone (TH) upon the $\mathfrak{Q}O_2$ of guinea pig thyroid expressed as a nomogram.

used. For example, 10 U of thyrotropic hormone will produce by itself an elevation in $\mathfrak{Q}O_2$ of 22% and this can be completely neutralized if 36γ% of thyroglobulin be added.

Obviously it is not possible to draw with complete accuracy a mathematical expression for a biological phenomenon in which so many factors are involved; yet it seems permissible, as a first approximation, to derive a nomogram for the prediction of results when known quantities of hormone act upon the test tissue, from such data as that shown in figure 1. This has been accomplished, in figure 2, by the simple expedients of smoothing both curves and reversing the one for thyroglobulin in such fashion that it begins where the other curve ends. The points of origin of the two curves have been connected by a horizontal line, upon which have been noted the values for percentage variation of $\mathfrak{Q}O_2$. The central point of this line represents zero deviation; points to the left, minus variations; and to the right, plus variations. The several lines shown connecting points in the upper and lower curves are the expression of the same experiments as shown in figure 1. Any line connecting points representing quantities of hormones which will completely neutralize each other will pass through the zero point of the $\mathfrak{Q}O_2$ line. If one wishes to predict the action of any combination of the two hormones he can do so by drawing the line between the appropriate points in the

upper and lower curves and reading the answer where the horizontal line is intersected by it; or knowing $\mathcal{Q}O_2$ and blood iodine concentration, he may estimate the concentration of thyroid stimulating hormone (TSH) in serum by drawing a straight line through the approximate points in the lower curve and the horizontal. TSH will be indicated at the point of intersection with the upper curve. Furthermore, inasmuch as basal metabolic rate is also, in all probability, a fair index of level of circulating thyroid hormone, values for it might be substituted in the lower curve of the nomogram, and an approximation, at least, of the hormonic balance could be derived, when one had determined the effect of a subject's serum on guinea pig thyroid $\mathcal{Q}O_2$ and had determined the subject's basal metabolic rate.

DISCUSSION

The thyroid-stimulating action of extracts of the anterior pituitary (thyrotropic hormone, or TSH) has been demonstrated both in animals of several species and *in vitro* by many investigators (1–33). It is also known that feeding or injecting thyroid hormone into animals (6, 25, 34–38) or human beings with goiter (39, 40) produces the histologic picture of a hypofunctioning gland. When the two hormones are given together *in vivo*, the action of the one neutralizes completely or partially the action of the other (20, 24, 25, 41, 42).

From these results, as well as those reported in the present paper, it is obvious that the pituitary can stimulate the thyroid. This is presumably a direct action of the pituitary hormone on its end-organ, the thyroid. It is also apparent that the thyroid hormone causes inhibition of the thyroid but this could be through the pituitary or directly. It is our opinion that the thyroid hormone inhibits both the anterior pituitary with respect to thyrotropic hormone production, and also the thyroid gland directly. That it inhibits the pituitary is suggested by the fact that Holweg and Junkmann (quoted by Reforzo, 25), found that thyroid administration to normal rats produces a decrease in the thyroid-stimulating power of their pituitaries. Reforzo (25), moreover, found that the weight of the thyroid glands of normal and of pituitarectomized guinea pigs is diminished when the animals are injected with extracts made from the pituitary glands of rats treated with thyroid by mouth. That the thyroid is also directly inhibited by its own hormone is indicated by the present work in which it has been shown that the *in vitro* addition of thyroid hormone to serum causes a fall in the oxygen consumption of the thyroid gland.

A schema of the pituitary-thyroid hook-up has been given in a previous paper from this clinic by Means (43). To carry the interpretation in graphic form further and to include the hypothesis of self-regulation by the thyroid, the series of schemata shown in figure 3 have been prepared. In schema *N*, of this figure, it is indicated that anterior pituitary (AP) stimulates the thyroid (T) by means of its hormone (TSH), shown by an arrow, which causes the thyroid to make its hormone (TH), also shown by an arrow, which is utilized by all the tissues of the body (O_2) for the maintenance of their rate of oxidation. Some of the thyroid hormone is inactivated and excreted, some remains in circulation and serves to inhibit both the thyroid gland directly and the pituitary. It is believed that the blood level of the hormone is kept relatively constant by this mechanism.

In figure 3, *A*, *B*, and *C* represent three steps in the response to a higher demand for thyroid hormone as in muscular work or exposure to cold. The increased demand for thyroid hormone causes a drop in its blood level (*A*) thus releasing the pituitary and thyroid glands from the inhibitory action of this hormone, with resulting increased production of thyroid hormone (*B*). If now the demand for thyroid hormone

falls there is left an excess of this hormone in the circulation, not utilized, which would inhibit the activity of the thyroid directly and indirectly by inhibiting the production of thyrotropic hormone by the pituitary gland (*C*). Presently the balance will return to that shown in schema *N*.

In figure 3, *D*, *E*, and *F*, the same mechanism is applied to explain the hypoactivity of the gland when thyroid hormone is administered to the organism. Such administration will increase the blood level of hormone (*D*), thus producing an increased

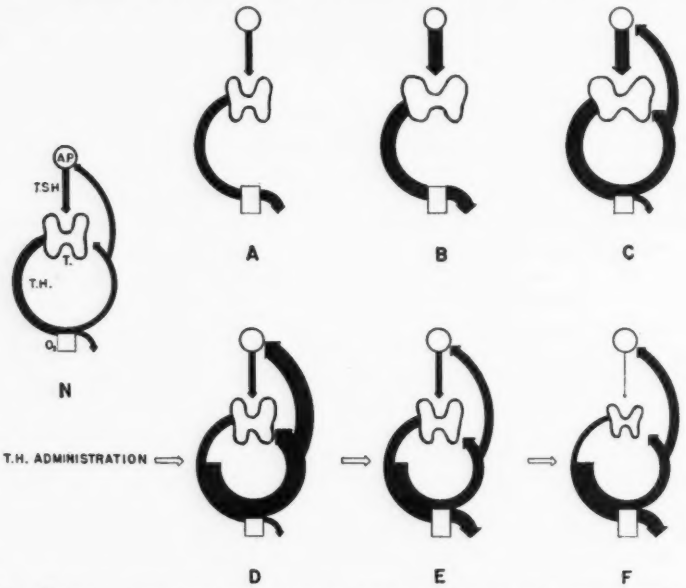

Fig. 3. SCHEMATA INDICATING PITUITARY-THYROID RELATIONSHIPS UNDER NORMAL CONDITIONS, *N*; under increased demand for thyroid hormone, *A*, *B* and *C*, and during the administration of thyroid hormone, *D*, *E* and *F*.

oxidation of all tissue cells (*E*) and an inhibition of both the thyroid gland and the pituitary gland (*F*).

SUMMARY

The $\mathcal{Q}O_2$ of the thyroid tissue of guinea pigs was determined by the Warburg technic. Human serum was used as a medium and increasing amounts of thyrotropic hormone and thyroid hormone were added, separately and simultaneously.

It was found that thyrotropic hormone increased the $\mathcal{Q}O_2$ of thyroid tissue and that thyroid hormone reduced it.

Thyrotropic hormone is inhibited *in vitro* by thyroglobulin. Its stimulating action is nullified if the right amount of thyroid hormone is added to the medium.

The hypothesis is advanced that circulating thyroid hormone acts as a regulator of thyroid gland activity. A rising level of thyroid hormone in the blood causes inhibition of the gland; a falling one removes inhibition, which in effect amounts to stimulation.

Reasons are given for believing that the inhibiting effect of thyroid hormone on thyroid tissue constitutes a regulating mechanism independent of the inhibiting effect of thyroid hormone on thyrotropic hormone production by the pituitary.

A nomogram is presented whereby, if the QO_2 of guinea pig thyroid immersed in serum and the blood organic iodine level or the basal metabolic rate of the subject be determined, an approximation of the thyrotropic hormone level of the blood may be reached.

I wish to thank Dr. J. H. Means for the facilities he has given me in his laboratory and for his supervision of my work.

REFERENCES

1. ALESCHIN, B.: *Rev. franc. d'endocrinol.* 4: 237. 1939.
2. ANDERSON, E. M., AND J. B. COLLIP: *Proc. Soc. Exper. Biol. & Med.* 30: 680. 1933.
3. ANDERSON, R. K., AND H. L. ALT: *Am. J. Physiol.* 119: 67. 1937.
4. ARON, M. *Compt. rend. Soc. de biol.* 102: 682. 1929.
5. ARON, M.: *Compt. rend. Soc. de biol.* 132: 544. 1939.
6. BELASCO, I. J., AND J. R. MURLIN: *Endocrinology* 28: 145. 1941.
7. BENEDICT, E. B., T. J. PUTNAM AND H. M. TEEL: *J.A.M.A.* 179: 489. 1930.
8. BAUMAN, E. J., AND D. MARINE: *Proc. Soc. Exper. Biol. & Med.* 29: 1220. 1932.
9. CANZELLI, A., AND D. RAPPORT: *Endocrinology* 22: 73. 1938.
10. COLLIP, J. B., H. SELYE, D. L. THOMPSON AND J. E. WILLIAMSON: *Proc. Soc. Exper. Biol. & Med.* 30: 590. 1933.
11. ELMER, A. W., B. GIEDOSZ AND M. SCHEPS: *Compt. rend. Soc. de biol.* 130: 309. 1940.
12. ETKIN, W.: *Proc. Soc. Exper. Biol. & Med.* 34: 508. 1936.
13. FOSTER, G. L., A. B. GUTMAN AND E. A. GUTMAN: *Proc. Soc. Exper. Biol. & Med.* 30: 1028. 1933.
14. HALPERN, C. A.: *Proc. Soc. Exper. Biol. & Med.* 32: 854. 1934.
15. HOUSSAY, B. A., A. BIASSOTTI AND A. MAGDALENA: *Rev. Soc. argent. de biol.* 8: 130. 1932.
16. KROGH, M., A. L. LINDBERG AND A. OKKELS: *Acta path. et microbiol. Scandinav.* 9: 37. 1932.
17. LOEB, L., AND R. B. BASSET: *Proc. Soc. Exper. Biol. & Med.* 26: 860. 1928.
18. LOEB, L., AND R. B. BASSET: *Proc. Soc. Exper. Biol. & Med.* 27: 490. 1929.
19. LOEB, L., AND H. FRIEDMAN: *Proc. Soc. Exper. Biol. & Med.* 29: 14. 1931.
20. LOEB, L., R. B. BASSETT AND H. FRIEDMAN: *Proc. Soc. Exper. Biol. & Med.* 28: 209. 1930.
21. LOEB, L., AND H. FRIEDMAN: *Proc. Soc. Exper. Biol. & Med.* 29: 172. 1931.
22. LOEB, L.: *Proc. Soc. Exper. Biol. & Med.* 29: 642. 1931.
23. LOEB, L.: *Proc. Soc. Exper. Biol. & Med.* 29: 1128. 1932.
24. LOEB, L.: *Endocrinology* 16: 129. 1932.
25. REFORZO MEMBRIVES, J.: Estudois sobre la relacion entre hipofisis y tiroides, El Ateneo, Buenos Aires, 1938.
26. RIDDLE, O., AND I. POLHEMUS: *Am. J. Physiol.* 98: 121. 1931.
27. SCHOCKAERT, J. A.: *Proc. Soc. Exper. Biol. & Med.* 29: 306. 1931.
28. SCHWARTZBACH, S., AND E. UHLENHUTH: *Proc. Soc. Exper. Biol. & Med.* 26: 152. 1928.
29. SILBERBERG, M.: *Proc. Soc. Exper. Biol. & Med.* 27: 166. 1929.
30. SMELSER, G. K.: *Proc. Soc. Exper. Biol. & Med.* 37: 388. 1937.
31. STARR, P., AND R. W. RAWSON: *Proc. Soc. Exper. Biol. & Med.* 35: 603. 1937.
32. ZAJIC, F.: *Compt. rend. Soc. de biol.* 118: 273. 1934.
33. ZUNZ, E., AND J. LA BARRE: *Compt. rend. Soc. de biol.* 118: 1622. 1935.
34. CORDONNIER, J.: *Proc. Soc. Exper. Biol. & Med.* 26: 637. 1928.
35. GRAY, S. H., AND L. LOEB: *Am. J. Path.* 4: 257. 1928.
36. GRAY, S. H., AND J. RABINOVITCH: *Am. J. Path.* 5: 485. 1929.
37. KUSCHINSKY, G.: *Arch. f. exper. Path. u. Pharmakol.* 162: 183. 1931.
38. UOTILA, U. U.: *Endocrinology* 26: 129. 1940.
39. RIENHOFF, W. F.: *Arch. Surg.* 41: 487. 1940.
40. RIENHOFF, W. F.: *Bull. Johns Hopkins Hosp.* 63: 538. 1941.
41. ARON, M., C. VAN CAULERT AND J. STAHL: *Compt. rend. Soc. de biol.* 107: 64. 1931.
42. LOESER, A., AND K. W. THOMPSON: *Endokrinologie* 14: 144. 1934.
43. MEANS, J. H.: (In press.)

THE RELATION OF THE THYROID AND THE PITUITARY GLANDS TO IODINE METABOLISM[1,2]

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THE RELATION BETWEEN THE PITUITARY and thyroid glands has been demonstrated by many workers since Loeb and Bassett (1) and Aron (2) more than a decade ago described a thyroid-activating principle of the pituitary gland. Since that time the pituitary has occupied a dominant rôle in our interpretation of thyroid physiology.

A definite reciprocal relationship of the thyroid to the pituitary is no less true, as illustrated by the morphologic changes in the pituitary glands of animals that have goiter (3) in cretinism (4) and after thyroidectomy (5). The increased output of urinary thyrotropic hormone after thyroidectomy (6) is also suggestive.

Knowledge regarding the rôle played by iodine in this system is necessary for complete understanding of these interrelationships; its undoubted integration with the physiology of the thyroid has been stressed by the early work of Marine and Williams (7). The part played by the pituitary gland in this integration must now be established. The work of Kuschinsky (8) and of Loeser and Thompson (9) and the more recently expressed opinion of Marine (10) gives the hypophysis a major rôle in the sphere of iodine metabolism as it relates to the function of the thyroid.

It has been postulated that the pituitary is influenced by the level of iodine in the body fluids (11) and that through the pituitary the thyroid and its integral activity in iodine metabolism are adjusted secondarily (12). If this holds true then the thyroid remains totally dependent on the pituitary and the basis for dysfunction of the thyroid and the metabolism of iodine must be looked for in the pituitary gland. Since our understanding of thyroid disease depends on a definite delineation of this relationship, an answer to this problem was sought.

METHODS OF STUDY

White rats were used; 16 male animals, 5 weeks of age, were selected and placed on a low iodine diet, described by Remington (13), containing approximately 15 micrograms of iodine per kg of food (14). Iodine in the form of potassium iodide was added to the drinking water so that 1 cc. of water contained 1.5 micrograms of iodine. The animals were maintained on this regimen for 5 weeks and then were hypophysectomized, the standard parapharyngeal approach being used. The completeness of the hypophysectomy was evaluated by the flattened growth curve, the testicular atrophy and microdissection of the hypophyseal region at necropsy. Hypophysectomy was

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[1] A portion of thesis submitted to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This study, combined with the paper entitled "Extrathyroidal iodine metabolism," was awarded the Van Meter Prize by the Association for the Study of Goiter and read before the meeting of the American Association for the Study of Goiter, Boston, Massachusetts, May 12 to 14, 1941.

[2] Work done at the Institute of Experimental Medicine under the direction of Dr. G. M. Higgins.

found to be complete in all but one animal which was excluded from the data in this study.

Immediately after operation the rats were divided into two groups of 8 animals each; both groups continued on the low iodine diet but the iodine supplement in the drinking water was withheld from one group and continued in the other. Fifty-five days after operation the experiment was terminated and the rats were killed with ether. Necropsy was performed immediately and the dissected tissues were carefully weighed and placed in 10% solution of formalin. The average acinar cell height was determined by a modification of the technic of Starr and Rawson (15). Every fifth section of the thyroid gland was mounted and stained (hematoxylin and eosin). From

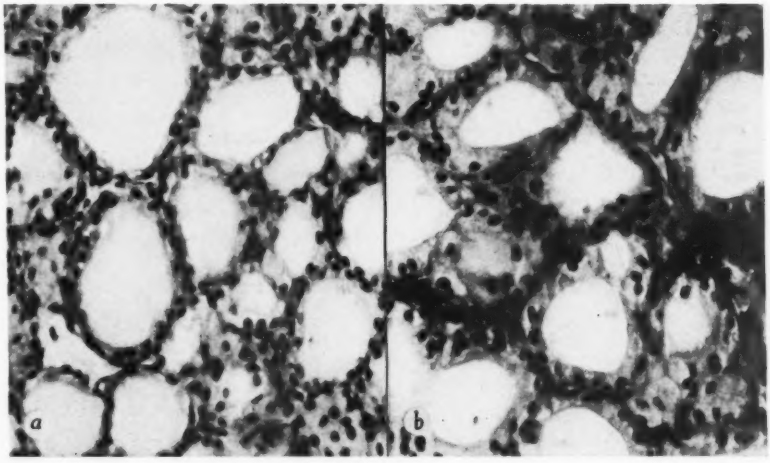

Fig. 1. SECTION OF THYROID GLAND OF INTACT ANIMAL RECEIVING (*a*) adequate iodine and (*b*) low iodine. ×325.

these sections the mean acinar cell height was determined by measuring 100 cells in well formed acini with an ocular micrometer, passing completely across each section of the gland.

The present study constitutes a corollary to a study of the effect induced when iodine was withheld from the intact and thyroidectomized animal (16). The group of intact animals in that study forms the control series for the observations presented in this paper. The source of animals, experimental conditions and management were identical, including the measurement of growth, food and water intake, and respiratory metabolism.[3]

Because of their volume it is impossible to include all the data. Therefore only an analysis of the thyroid tissue of the different groups is presented.

EXPERIMENTAL DATA

The general condition of the animals was good. One animal of the hypophysectomized, low iodine group died on the 40th postoperative day and is not included in this study.

[3] The experimental period was 65 days for the intact animal group and 55 days for the hypophysectomized group.

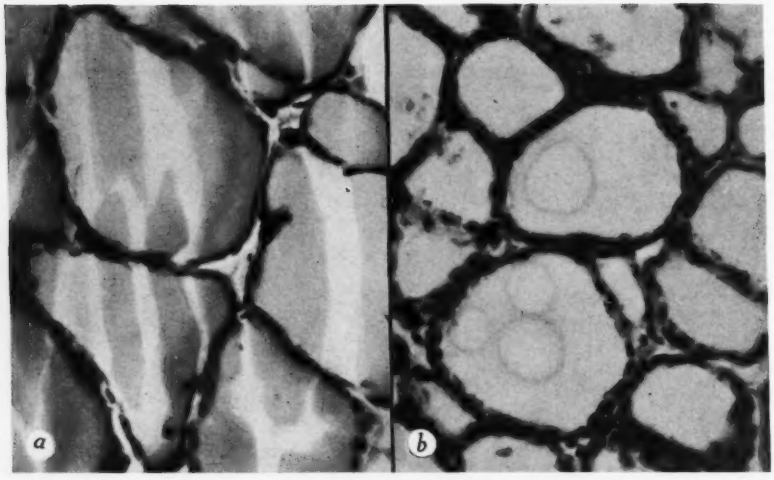

Fig. 2. SECTION OF THYROID GLAND OF (*a*) hypophysectomized animal receiving iodine supplement and (*b*) one on low iodine. ×310.

Gross observations of the thyroid gland. In gross appearance the thyroid glands of the intact animals receiving a supplement of iodine were of normal, pinkish color. They were moderately soft and their vascularity was normal. The thyroid glands of the intact animals on a low intake of iodine were enlarged (average 110% by weight) as compared with those of the control group; they were beefy red; the tissue was friable and exceedingly vascular and the major vessels to the gland were greatly enlarged.

The thyroid glands of the hypophysectomized animals receiving a supplement of

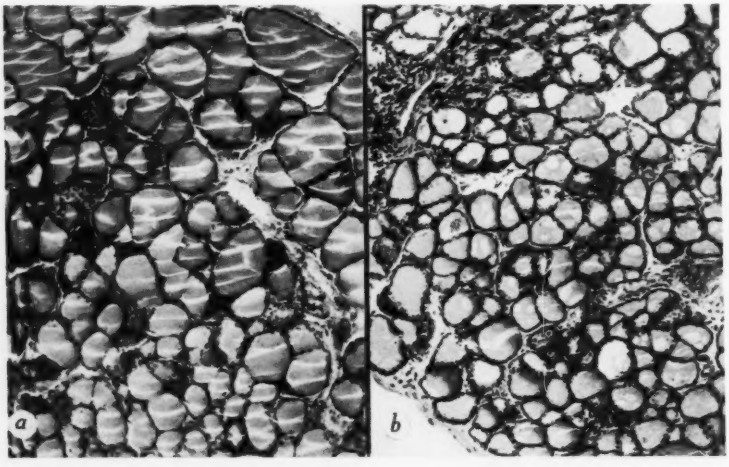

Fig. 3. LOWER MAGNIFICATION (×90) OF SECTIONS OF THYROID GLAND OF HYPOPHYSECTOMIZED ANIMAL (*a*) on iodine supplement and (*b*) on low iodine. In the low iodine group the acini are somewhat smaller and there is greater vascularity of the gland than in the group on iodine supplement.

iodine were smaller than normal, light buff and somewhat translucent. They were hard and cartilaginous under the dissecting forceps and were extremely avascular; the blood vessels supplying them were small and thin and contained little blood. In definite contrast to the foregoing were the glands of the hypophysectomized group maintained on a low intake of iodine. These glands were larger (average 34.8% by weight) were distinctly pink and were not translucent. They were softer and more vascular than those of the group of hypophysectomized animals which received a supplement of iodine but they were less vascular than those of the intact control animals. The contributing vessels to the glands were slightly smaller than normal.

Microscopic observations. The thyroid glands of the intact animals receiving adequate iodine (fig. 1*a*) appeared on microscopic examination to be normal in all re-

TABLE 1. SUMMARY OF EXPERIMENTAL DATA

	Intake of iodine	Blood supply	Average wt. of thyroids	Average acinar cell ht.
			mg.	*μ*
Intact animals	Iodine supplement	Normal	13.0	6.0
	Low iodine	Marked increase	27.3 (+110%)[1]	10.7 (+78%)[1]
Hypophysectomized animals	Iodine supplement	Marked decrease	6.9	1.5
	Low iodine	Slight decrease	9.3 (+35%)[1]	2.6 (+73%)[1]

[1] Percentage deviation from that of group receiving iodine.

spects; the follicles were filled with well stained colloid, the epithelium was of normal height (average 6.0*μ*), there was a normal amount of intrafollicular stroma and there was no increase in the blood supply. The glands of the intact animals receiving a low intake of iodine presented an entirely different appearance (fig. 1*b*). The colloid was poorly stained or absent; the cells of the acinar epithelium were heightened (average 10.7*μ*), vacuolated and at some points invaginated. There were also an increase in the intrafollicular stroma and a marked increase in the vascularity of the glands.

Sections of the thyroid glands of hypophysectomized animals are shown in figures 2 and 3. Examination of the thyroids of the hypophysectomized group receiving a supplement of iodine (fig. 2*a* and 3*a*) revealed the type of gland usually described as associated with anterior pituitary insufficiency. The follicles were well filled with deeply stained colloid; the acinar cells were flattened (average 1.5*μ*) and resembled a squamous epithelium in many places. A minimal amount of fibrous tissue was present and the vascularity of the glands was much less than in the normal animal. These glands correspond to what has been called 'resting' grandular tissue. Not so, however, were the thyroid glands of the hypophysectomized group receiving an inadequate iodine supply (fig. 2*b* and 3*b*). The amount of colloid within the follicles was approximately the same as in the preceding group, but the acinar epithelium was distinctly elevated (average height 2.6*μ*) above that of the hypophysectomized animals which did receive iodine and was for the most part cuboidal in type; the nuclei were more deeply stained and larger; there was a moderate amount of intrafollicular fibrous

stroma and the blood supply to the follicles was significantly greater than that of the glands in the preceding group. These glands gave the appearance of being stimulated.

COMMENT

Two significant facts were revealed in the foregoing data. First, the thyroid gland was able to receive and respond to the stimulus of low iodine in the absence of the pituitary. Second, the elevation of the height of the thyroid epithelium which occurred in the hypophysectomized animals exposed to the stimulus of low iodine was almost exactly proportional to the elevation of the thyroid epithelium which occurred in the intact animals under the same stimulus. The response in the hypophysec-

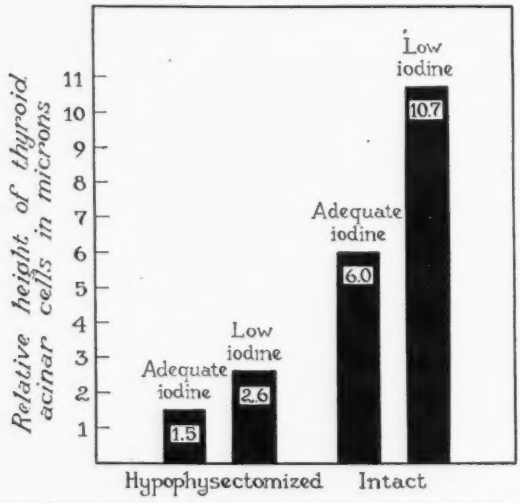

Fig. 4. Graph showing relative mean heights of thyroid acinar cells of groups summarized in table 1.

tomized animals to low iodine was an increased thyroid epithelial height of 73%; that of the intact animals was 78% (table 1). This relation suggests that the reaction involved in this response is independent of the pituitary gland. The proportional heights are shown in figure 4 and represent the mean acinar cell height of the thyroid epithelium, in microns, of the 4 experimental groups of animals.

SUMMARY AND CONCLUSIONS

Observations on the thyroid glands of a series of hypophysectomized and intact animals maintained on high and low iodine diets have been presented.

The thyroid glands of intact animals receiving an inadequate supply of iodine showed gross and histologic evidence of hypertrophy as compared with those of the intact animals receiving adequate iodine.

The thyroid glands of the intact animals responded to the stimulus of a low intake of iodine with an increase in weight, increase in height of acinar epithelium and an increased vascularity.

The thyroid glands of the hypophysectomized animals responded to the stimulus of a low intake of iodine in a similar manner to that of the intact animals with an in-

crease in weight, increase in the height of the acinar epithelium and an increased vascularity. The increase in height of the acinar epithelium in the hypophysectomized group was proportionately the same as that of the intact group.

It is concluded from the evidence presented here that the thyroid gland is able to respond to the stimulus of low iodine in the absence of the pituitary.

REFERENCES

1. Loeb, L., and R. B. Bassett: *Proc. Soc. Exper. Biol. & Med.* 26: 860. 1929.
2. Aron, M.: *Compt. rend. Soc. de biol.* 102: 682. 1929.
3. Nelson, Rose C., and J. Warkany: *Proc. Soc. Exper. Biol. & Med.* 39: 66. 1938.
4. Nièpce, B.: Traité du goître et du crétinisme, suivi de la statistique des goîtreux et des crétins dans le bassin de l'Isère en Savoie, dans les départements de l'Isère, des Hautes-Alpes et des Basses-Alpes. Paris, J.-B. Baillière, vol. 10, 501 pp. 1851.
5. Severinghaus, A. E., G. K. Smelser and Helen M. Clark: *Proc. Soc. Exper. Biol. & Med.* 31: 1127. 1934.
6. Starr, P., R. W. Rawson, R. E. Smalley, E. Doty, and Helen Patton: *West. J. Surg.*, 47: 65. 1939.
7. Marine, D., and W. W. Williams: *Arch. Int. Med.* 1: 349. 1908.
8. Kuschinsky, G.: *Arch. f. exper. Path. u. Pharmakol.* 170: 510. 1933.
9. Loeser, A., and K. W. Thompson: *Internat. Clin.* 45. 3: 149. 1935.
10. Marine, D.: *J.A.M.A.* 104: 2250. 1935.
11. Salter, W. T.: The Endocrine Function of Iodine. Harvard University Press, p. 131. Cambridge, Massachusetts, 1940.
12. Loeser, A., and K. W. Thompson: *Endokrinologie* 14: 144. 1934.
13. Remington, R. E.: *J. Nutrition* 13: 223. 1937.
14. Remington, R. E.: Personal communication to the author.
15. Starr, P., and R. W. Rawson: *Proc. Soc. Exper. Biol. & Med.* 35: 603. 1937.
16. Chapman, A.: Unpublished data.

EXTRATHYROIDAL IODINE METABOLISM[1,2]

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IODINE HAS BEEN IDENTIFIED with the physiology of the thyroid gland since the findings of Coindet (1) and Chatin (2). The studies of Baumann (3) Marine (4), Kendall (5) and recently many others have contributed to our knowledge of iodine metabolism. These studies have focused attention on the intimate relation that exists between iodine and the thyroid. The effects of either the administration or restriction of iodine, hitherto observed are, for the most part, effects on the thyroid gland or those which imply the participation of thyroid tissue. Recently the extrathyroidal production of thyroxine or thyroxine-like substances from the iodization of protein has been accomplished. In view of this finding, a study of the effect of iodine on the completely thyroidectomized animal should be significant.

In an investigation of certain thyroid-pituitary relationships it was observed that a group of thyroidectomized animals on a low iodine diet sustained a progressive loss of weight, while a control group of thyroidectomized animals receiving a small supplement of iodine appeared to be in good general condition and progressively gained weight. This observation seemed worthy of further study, the results of which form the basis of this report.

METHODS OF STUDY

For this investigation albino rats were used. Forty-two male animals, aged 7 weeks and weighing approximately 165 gm., were selected and divided into two groups. Twenty-four rats on which thyroidectomy was not performed formed one group and 18 thyroidectomized rats formed a second group. Thyroidectomy was done by a uniform technic with the rats under ether anesthesia and was complete in all animals but one in which a small amount of thyroid tissue was observed at necropsy.

All animals were housed in steel cages equipped with wire-mesh bottoms; each cage was occupied by 4 to 6 animals. All animals received the same goitrogenic low iodine diet described by Remington (6) which contains approximately 15 micrograms of iodine per kg. of food (7). It was prepared in biscuit form and given ad libitum in wire mesh baskets which were removable from the cage and, with their contents, easily weighed. All animals had free access to distilled water.

The intact and the thyroidectomized animal groups were each divided in half; 12 intact and 9 thyroidectomized animals each formed a group which received an iodine supplement. This was administered in the drinking water, 1.5 micrograms of iodine as potassium iodide per cc. of water. The iodine intake in these two groups averaged 34 and 27 micrograms respectively per rat per day with less than 5% difference in the two groups calculated on a basis of average body weight.

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[1] A portion of thesis submitted to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy. This study, combined with the essay "The relation of the thyroid and the pituitary glands to iodine metabolism," was awarded the Van Meter Prize by the Association for the Study of Goiter.

[2] Work done at the Institute of Experimental Medicine under the direction of Dr. G. M. Higgins.

Thus four groups of animals were arranged. Two of these were of intact animals, 12 in each group, one receiving an iodine supplement adequate for the intact animal, and one maintained on a low iodine, goitrogenic diet and not receiving any iodine supplement. The other two groups were composed of 9 thyroidectomized animals each, one receiving approximately the same iodine intake as the group of intact animals and one receiving the low iodine diet without any supplement.

Body weights were recorded every third day and the consumption of food and water was measured and recorded daily.

Respiratory metabolism was determined twice during the course of the experiment. For this purpose a closed chamber containing an absorbent of carbon dioxide

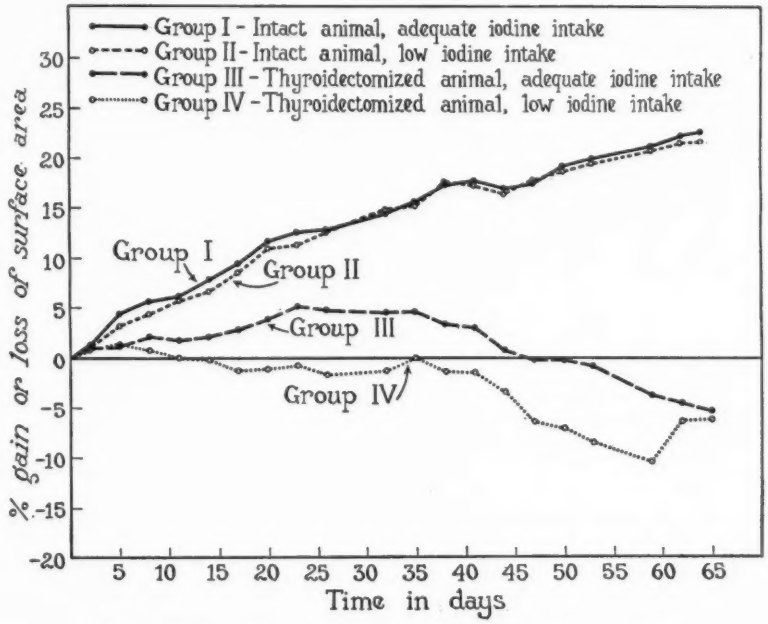

Fig. 1. Growth curves expressed as percentage changes in surface area.

was devised (8). This chamber, in which the rat was placed, was submerged in a constant temperature bath (28°C.) and the volume of oxygen consumed was observed over a certain period. Food was withheld for 18 hours preceding the determination and at least three consecutive readings were made for each animal in estimating the basal metabolic rate.

The animals were killed for necropsy with ether; the tissues were removed immediately, weighed and fixed in 10% solution of formalin.

EXPERIMENTAL DATA

The data have been calculated and expressed in terms of unit body weight and body surface area because of the diverging weights of the different groups. Thus the food intake of a group of rats averaging 200 gm. was compared properly with the food intake of a group averaging 150 gm.

Food and water intake and changes in body weight were observed from the 4th to the 69th postoperative day, thus constituting a 65-day experimental period.

The changes in surface area of these animals (fig. 1) were based on the percentage change in weights from the onset of the experiment. Difference in the level of iodine intake made little change in the rate of growth of intact animals but in thyroidectomized animals differences were observed. Those on restricted iodine intake lost weight and surface area more rapidly than the animals receiving the iodine supplement. The terminal rise of the growth curve (fig. 1) in the thryoidectomized animals

TABLE 1. CHANGES IN WEIGHT AND SURFACE AREA DURING THE EXPERIMENTAL PERIOD

Animals	Groups	Actual gain or loss over total period[1]		Average gain or loss/rat/day	
		Weight	Surface	Weight	Surface
		gm.	*sq. cm.*	*gm.*	*sq. cm.*
Intact	I Adequate iodine	+930	+796	+1.21	+1.03
	II Low iodine	+898	+770	+1.17	+1.00
Thyroidectomized	III Adequate iodine	− 73	− 83	−0.13	−0.15
	IV Low iodine	−243	−251	−0.45	−0.47

[1] Changes in animals that died during course of experiment are included.

on low iodine intake is explained by the death of low weight members of this group, whereas the animals whose percentage loss of surface area was less survived.

The actual changes in weight and surface area of the four groups (table 1) included the changes occurring during the life of the rats which died during the course of the experiment. These figures represent the net performance of the groups more accurately than does the graph of percentage changes but they do not show the progressive changes so well. Again there was noted very little difference in the changes in weight or surface area in the two groups of intact animals receiving low and adequate iodine intake. But of the thyroidectomized animals, those receiving the low iodine diet without iodine supplement suffered a threefold loss of weight and surface area in comparison with those animals in the thyroidectomized group receiving an iodine supplement.

The food intake and the gain or loss of surface area in proportion to the food consumed were computed (table 2). The latter comparison represents a measure of food utilization. The food intake and its utilization by the two groups of intact animals were approximately the same. However, among the thyroidectomized animals, although those receiving a low iodine intake consumed almost as much food as the controls receiving iodine, yet they lost more than three times as much weight and surface area. That is, the thyroidectomized animals that received iodine utilized their food better than the thyroidectomized animals which did not receive an iodine supplement.

TABLE 2. INTAKE OF FOOD. RELATION TO CHANGES IN SURFACE AREA

Animals	Groups	Food intake for group		Gain or loss of S.A. /gm. of food intake/24 hr.
		Total	Average/sq. cm. of S.A./24 hr.	
		gm.	*gm.*	*sq. cm.*
Intact	I Adequate iodine	13,162	0.0534	+0.061
	II Low iodine	13,313	0.0541	+0.058
Thyroidectomized	III Adequate iodine	5,762	0.0383	−0.014
	IV Low iodine	4,818	0.0327	−0.052

A study of water intake in relation to surface area, to change in surface area and to the intake of food showed (table 3) that the water consumption in intact animals on either low or adequate iodine intake was not altered significantly. The water intake in thyroidectomized animals on low iodine intake, however, was significantly greater than in the thyroidectomized animals receiving iodine. This was evident in relation to surface area as well as to the food intake. For every gram of food taken by the thyroidectomized animals receiving supplemental iodine, 1.7 cc. of water was consumed, while the thyroidectomized group on low iodine consumed 2.4 cc. of water for each gram of food. The expression of loss of surface area in relation to water consumed indicates that the loss was approximately proportional to the loss calculated on a basis of food intake; that is, even though the water consumption was greater, the loss in weight and surface area progressed. It is probable that the changes in weight and sur-

TABLE 3. INTAKE OF WATER. RELATION TO CHANGES IN SURFACE AREA

Animals	Groups	Intake of water			Gain or loss of S.A./cc. of water
		Total	Average/sq. cm. of S.A./24 hr.	Ratio, to intake of food[1]	
		cc.	*cc.*		*sq. cm.*
Intact	I Adequate iodine	17,980	0.070	1.3	+0.044
	II Low iodine	17,461	0.068	1.3	+0.038
Thyroidectomized	III Adequate iodine	10,050	0.065	1.7	−0.008
	IV Low iodine	11,424	0.078	2.4	−0.022

[1] Ratio of cubic centimeters of water to grams of food.

face area were independent of the water intake and that the animal had diuresis and not retention of water. The relative differences in the amounts of food and water consumed and in the change in surface area of the 4 groups of animals are shown in figure 2.

The metabolic rate of the animals was determined on the 49th and 64th days of the experimental period; a slight decrease in the oxygen consumption occurred in the second determination. The average of the two determinations was selected as a basis for comparison of the metabolic rates in the 4 groups (table 4). A slight increase (6%) in the average basal metabolism of the intact (goitrous) animals receiving low amounts of iodine over that of the intact animals receiving adequate amounts of iodine may not be of sufficient magnitude to be significant. However, a real difference between

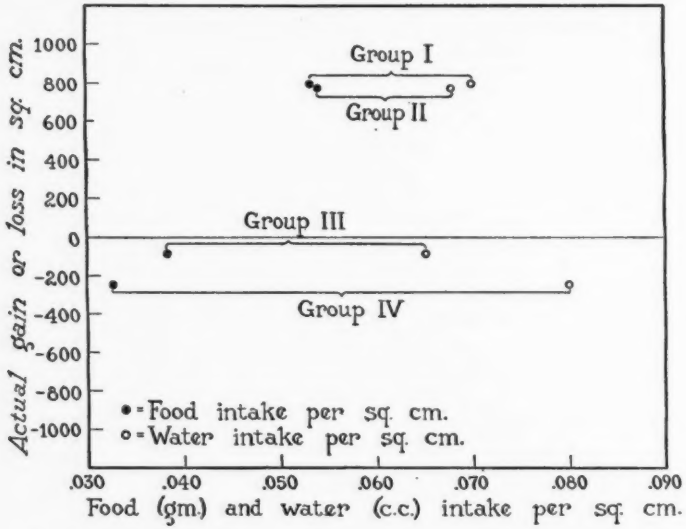

Fig. 2. Relation of intake of food and water to change in surface area. The length of the brackets indicates the ratios of food to water intake in the groups.

the mean metabolic rate of the two thyroidectomized groups, with and without the iodine supplement, was observed. If the one animal in the thyroidectomized group which did not receive any iodine supplement and which showed regenerated thyroid tissue at necropsy is excluded, then the basal metabolic rate for this group was depressed 27% below that of the thryoidectomized control group receiving the iodine supplement.

The caloric value of the food intake of the groups was calculated (table 4). However, as this was not consumed under basal conditions the comparison with the caloric output is only relative.

Weights and measurements of necropsied material showed (table 5) that the thyroid glands of the goitrous animals on low iodine intake weighed about twice that of the group receiving adequate iodine intake. The average height of the acinar cells was determined by a modification of the method described by Starr and Rawson (9). Every fifth section of the thyroid gland was mounted and stained (hematoxylin and eosin). From these sections the mean acinar cell height was determined by measuring 100

TABLE 4. CALORIC INTAKE AS FOOD, BASAL CALORIC OUTPUT (CALCULATED FROM OXYGEN CONSUMPTION) AND PERCENTAGE DEVIATION OF METABOLIC RATES FROM THOSE OF CONTROL GROUPS

Animals	Groups	Average caloric intake, output/sq. m./24 hr.		Deviation of B.M.R. from normal	
		Intake	Output B.M.R.	Basis 1,032	Basis 588
		Cal.	*Cal.*	%	%
Intact	I Adequate iodine	1,922	1,032	0	
	II Low iodine	1,948	1,090	+6	
Thyroidectomized	III Adequate iodine	1,379	588	−43	0
	IV Low iodine	1,177	482 (432)[1]	−53 (−58)[1]	−18 (−27)[1]

[1] One animal with regenerated thyroid tissue (rate −30) was excluded.

cells in well formed acini with an ocular micrometer, taking care to pass entirely across each gland section. The average cell height of the goitrous, low iodine group was 78% higher than that of the control group receiving an iodine supplement to the diet.

The weights of the pituitary glands were approximately the same in all groups but in proportion to the body weight the glands of the thyroidectomized groups were larger. No significant difference in the average weight of the pituitary gland was noted between the low and adequate iodine groups in either the intact or thyroidectomized animals. The weights of the adrenal glands were not significantly different in the groups if considered on a basis of body weight.

The cytologic characteristics of the pituitary glands of this series are being studied; the results will be reported at a later date.

TABLE 5. AVERAGE WEIGHTS AND MEASUREMENTS OF THYROID, PITUITARY AND ADRENAL GLANDS

Animals	Groups	Wt. of thyroid gland	Ht. of thyroid acinar cell	Wt. of pituitary	Wt. of adrenal
		mg.	*μ*	*mg.*	*mg.*
Intact	I Adequate iodine	13.0	6.0	7.1	15.0
	II Low iodine	27.3	10.7	7.7	16.4
Thyroidectomized	III Adequate iodine	—	—	7.3	11.6
	IV Low iodine	—	—	7.2	11.7

Sections of the thyroid glands (fig. 3) showed normal epithelium, colloid content and vascularity in the groups receiving an iodine supplement. The enlarged glands from the goitrous group had an increased acinar cell height with some fragmentation and vacuolization of the cells; the colloid was thin or absent, poorly stained and vacuolated; there was greatly increased vascularity of the tissue.

COMMENT

It is evident that iodine played some rôle in the body economy of the thyroidectomized rats. The presence of thyroid tissue in the intact animals permitted utilization of the small amount of iodine available in the low iodine diet. To accomplish this the thyroid hypertrophied, thereby maintaining the growth and well-being of the organism, as shown by the growth curves of the two groups of intact animals.

The thyroidectomized animals, on the other hand, deprived of tissue especially

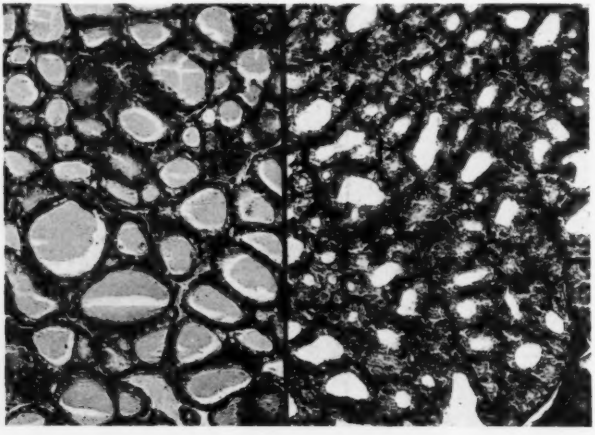

Fig. 3. Sections of thyroid glands of normal (*left*) and goitrous (*right*) animals.

adapted to the processing and utilization of iodine, suffered a change in body growth and function, even in the presence of a generous supply of iodine. But, with the double handicap of loss of specialized tissue and of a diminished supply of iodine the bodily functions were depressed further below normal levels and the organism showed evidence of severe physiologic distress. There was a definite elevation of the metabolic plane in the thyroidectomized animals receiving iodine which was reflected in the growth curves.

The possibility that iodine may have an effect on vital processes in the absence of thyroid tissue was emphasized by the work of Swingle (10), Hoskins and Hoskins (11), Allen (12) and others who showed that iodine would stimulate metamorphosis in thyroidectomized amphibians. Further evidence that iodine may affect growth processes in the absence of thyroid tissue was shown by Silberberg and Silberberg (13), who reported stimulation of growth of bone in immature thyroidectomized guinea-pigs by the administration of iodine. This resulted in a parallel to the growth of bone caused by the administration of thyroxine to thyroidectomized animals.

In the intact animal administration of iodine has been reported to enhance growth (14, 15) as well as to be without effect on growth (16, 17, 18); given to the lactating female rat iodine has been shown (19) to accelerate growth of the suckling young.

The increased utilization of food by the thyroidectomized animal which accompanied the administration of iodine is not understood. It may be a reflection of increased efficiency of the bodily processes which seems to be induced by administration of iodine to the thyroidectomized animal. Likewise, the difference in the water requirements of the two thyroidectomized groups which accompanied a difference in their iodine intake is not explained readily. However, subsequent work indicates that the hypophysis may play a role in these relationships.

The metabolic rate of the two thyroidectomized groups of animals also emphasizes the effect of iodine on oxygen consumption in the absence of thyroid tissue. A generous supply of iodine to the thyroidless animals raised the metabolic rate significantly above that of the thyroidectomized animals receiving little available iodine. On the other hand, the low iodine intake in the intact animal did not lower the metabolic rate (during the experimental period). This would tend to emphasize the buffer action of thyroid tissue to sustain bodily function under stress of inadequate iodine supply as compared with the relatively inefficient extrathyroidal mechanism for the utilization of iodine. However, a definite influence on respiratory metabolism was effected by the level of iodine intake in the thyroidectomized animal.

Inoue (20) produced a significant elevation of the metabolic rate by the administration of iodine to totally thyroidectomized guinea-pigs as well as to those from which seven-eighths of the thyroid had been removed. In animals that had a greater amount of remaining thyroid tissue a fall of the metabolic rate was produced by iodine. Siebert (21) reported a fall of oxygen consumption in the thyroidectomized guinea-pig with the administration of iodine. This may be explained by the presence of regenerated thyroid tissue which was found at necropsy in the majority of his animals.

Marine, Deutch and Cipra (22) did not observe a change in the metabolic rate of intact rabbits after the administration of iodine. This is not comparable, however, to the effect of iodine on the thyroidectomized animal in which there exists a severe need for thyroid processed iodine. Webster (23) induced a definite rise in oxygen consumption by the administration of iodine to goitrous patients who had low metabolic rates.

Three possible mechanisms have been considered to account for the effects produced by iodine in the absence of thyroid tissue. *a*) The increased iodine intake may influence some physiologic mechanism not related directly to thyroid function (for example, calcium metabolism). This is unlikely. *b*), Iodine may have some specific effect on the body cells. *c*), A thyroxine-like substance may be produced in the body tissues when iodine is available to them.

The latter consideration seems the most probable in the light of recent work. Abelin (24), Ludwig and von Mutzenbecher (25), Salter and Lerman (26), Block (27) and others have demonstrated the production of thyroxine, diiodotyrosine and calorigenic protein similar to thyroglobulin by the iodization of organic material in vitro.

It is possible that iodine in the thyroidless animals acts in a similar manner to that of iodine on organic matter in vitro, possibly aided by enzyme action not necessarily confined to thyroid tissue. This effect of iodine in the thyroidectomized animal, then, may represent the action on the tissues of a certain concentration of iodine, thus elaborating a thyroxine-like substance.

If an extrathyroidal utilization of iodine exists, then its function in disease associated with iodine metabolism may be very significant; this would apply especially to hypothyroid states. The untoward effects of iodine lack in the thyroidless animal suggest that an absolute iodine deficiency would impose a more severe strain on the organism and that life itself may not be compatible with the complete absence of iodine.

SUMMARY AND CONCLUSIONS

To study the effects of adequate and low iodine intake on the rat, groups of intact and thyroidectomized animals were prepared. Both groups received a low iodine diet. For control groups, half the animals in each group received an iodine supplement to the diet.

The growth curves of the intact and thyroidectomized animals receiving a low iodine diet and a supplemented iodine diet were studied. The level of low and high iodine intake did not alter greatly the changes of weight and surface area in the intact animals. But in the thyroidectomized animal groups the iodine intake significantly affected the changes of weight and surface area. Those animals receiving an iodine supplement lost much less weight and surface area than those on the restricted iodine intake.

The iodine intake had little effect on the food consumption or food utilization in the intact animals. But in the thyroidectomized animals a slightly increased food intake and a greatly increased food utilization occurred in the group receiving the higher iodine intake.

Water intake also was approximately the same in the two intact animal groups receiving a low and an adequate iodine intake. But the thyroidectomized group on low iodine consumed a significantly greater quantity of water than the thyroidectomized group receiving an iodine supplement.

The metabolic rate of the intact animal was not altered greatly by a difference in iodine intake. But in the thyroidectomized animals, those receiving the supplemental iodine had a significantly higher rate than those not receiving any supplement.

A difference in iodine level in the diets of thyroidectomized rats significantly altered the changes in weight and surface area, the water intake, the food utilization and the metabolic rate. These changes were not induced in the intact animals by deprivation or administration of iodine.

From the data presented it is concluded that iodine may play a rôle in body metabolism in the absence of thyroid tissue, possibly by the production of a thyroxine-like substance in the tissues.

REFERENCES

1. Coindet, C.: *Ann. de chim. et phys., Par.* 15: 49. 1820.
2. Chatin, A.: *Compt. rend. Acad. d. sc.* 36: 652. 1853.
3. Baumann, E.: *Ztschr. f. physiol. Chem.* 21: 319. 1895–1896.
4. Marine, D.: *J. Infect. Dis.* 4: 417. 1907.
5. Kendall, E. C.: *J.A.M.A.* 64: 2042. 1915.
6. Remington, R. E.: *J. Nutrition* 13: 223. 1937.
7. Remington, R. E.: Personal communication to the author.
8. Chapman, A.: Unpublished data.
9. Starr, P., and R. W. Rawson: *Proc. Soc. Exper. Biol. & Med.* 35: 603. 1937.
10. Swingle, W. W.: *Endocrinology* 2: 283. 1918.
11. Hoskins, E. R., and M. M. Hoskins: *Endocrinology* 4: 1. 1920.
12. Allen, B. M.: *Biol. Bull.* 36: 405. 1919.
13. Silberberg, M. and Ruth Silberberg: *Arch. Path.* 28: 846. 1939.
14. Kochmann, M.: *München med. Wchnschr.* 78: 565. 1931.
15. Mačela, I.: (Abst.) *J.A.M.A.* 85: 1926, 1925.
16. Remington, R. E., and J. W. Remington: *J. Nutrition.* 15: 539. 1938.
17. Cameron, A. T., and J. Carmichael: *J. Biol. Chem.* 45: 69. 1920.
18. Mendel, L. B., and H. B. Vickery: *Proc. Soc. Exper. Biol. & Med.* 27: 806. 1930.
19. Maurer, E., and S. Diez: *Biochem. Ztschr.* 182: 291. 1927.
20. Inoue, Seizaburo: *Arch. f. klin. chir.* 197: 155. 1939.
21. Siebert, W. J.: *Proc. Soc. Exper. Biol. & Med.* 29: 1146. 1932.
22. Marine, D., M. Deutch and Anna Cipra: *Proc. Soc. Exper. Biol. & Med.* 24: 657. 1927.
23. Webster, B.: Tr. Am. A. Study Goiter p. 424. 1938.
24. Abelin, I.: *Klin. Wchnschr.* 13: 940. 1934.
25. Ludwig, P., and P. von Mutzenbecher: *Ztschr. f. physiol. Chem.* 258: 195. 1939.
26. Salter, W. T., and J. Lerman: *Endocrinology* 20: 801. 1936.
27. Block, P., Jr.: *J. Biol. Chem.* 135: 51. 1940.

STUDIES ON THE HISTOLOGY AND PHYSIOLOGY OF THE PITUITARY OF RATS TREATED WITH PROGESTERONE[1]

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SEVERAL INVESTIGATORS (1–3) have reported that crystalline corpus luteum hormone or synthetic progesterone do not prevent castration changes which occur in the pituitary gland of rats following gonadectomy. Hohlweg (1), for example, injected castrated adult and immature female rats with crystalline corpus luteum hormone. The adult animals received 540 γ daily for 15 days, the immature animals as high as 40 γ daily for 16 days. Albrieux et al. (2) used castrated young male rats to which they administered a purified corpus luteum preparation at levels of ⅛ to ½ RB. U. daily for 15 days. Fels (3), without giving data, stated that progesterone was incapable of preventing castration effects in the pituitaries of spayed rats. In contrast to these observations Brooksby (4) reported that 0.5 mg. of progesterone daily for 10 days inhibited castration changes in the hypophyses of 3-month-old female rats.

Relatively large doses of progesterone have been demonstrated to have an inhibiting action on the output of hypophyseal gonadotropic hormone. Selye et al. (5) found that daily administration of 4 mg. of progesterone to normally cyclic rats caused immediate cessation of cycles. Phillips (6) likewise was uniformly able to interrupt estrous cycles in rats by means of progesterone in doses of 1.5 to 4 mg. daily. In rabbits Makepeace et al. (7) inhibited ovulation by the daily administration of 1 to 5 mg. of progesterone for 5 days. Burrows (8) found that pituitaries of normal young female rats which had received 5 mg. of progesterone produced less ovarian stimulation when implanted in mice than did the glands of control rats. Herlant (9) obtained variable results by the implantation method with pituitaries of rats receiving progesterone. Hypophyses from rats treated with 0.35 mg. or 0.5 mg. daily for 10 or 15 days contained less gonad-stimulating hormone than those of animals injected with 1 mg. daily for 4 days. The studies of Biddulph et al. (10) on immature parabiotic rats led them to conclude that 1 mg. of progesterone daily is required to prevent gonadotropic hyperactivity of the pituitary in the castrated twin. A recent interpretation of the factors concerned in the estrous cycle of the rat involves participation of the corpus luteum hormone (11). According to this theory progesterone inhibits the release of LH from the pituitary.

The present study was undertaken in an attempt to correlate morphological and physiological changes in the pituitary glands of rats consequent on injections of progesterone.

MATERIAL AND METHODS

The effect of progesterone[2] on the pituitary has been studied in nonparabiotic castrated rats and in normal or castrated rats parabiotically joined with hypophysectomized male or female partners.

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[1] This work was aided in part by Works Project Administration No. 665-51-3-225.
[2] The progesterone used in this work was supplied by Dr. Erwin Schwenk, Schering Corporation, Bloomfield, New Jersey.

Essential data on the experiments involving injections of progesterone in nonparabiotic castrated rats are given in table 1. Adult male and female animals were used, and injections of progesterone were begun either at 1 day or at 224 days after castration. Autopsies were performed 1 day after the final injection. Each dose of hormone was dissolved in 0.2 cc. of peanut oil, and injections were made subcutaneously. In the females injections were begun 1 day after spaying and continued daily for 12

TABLE 1. PROGESTERONE INJECTIONS IN CASTRATED MALE AND FEMALE RATS

Rat No.	Daily Dose of Progesterone in 0.2 cc. oil (mg.)	Total Number of Injections	Injections Begun, Days after Castration	Weight of Pituitary at Autopsy (mg.)
		Females		
22	3	12	1	11.2
24	2	12	1	8.5
25	1	12	1	8.8
26	½	12	1	6.4
	Castrate control	(1 rat)		9.8
	Normal control	(1 rat)		10.1
23	3	21	1	11.6
28	2	21	1	11.0
32	1	21	1	10.6
30	½	21	1	8.2
	Castrate control	(Av. 2 rats)		11.3
	Normal control	(Av. 3 rats)		12.6
		Males		
38	3	11	1	9.2
43	2	11	1	8.2
	Castrate control	(1 rat)		8.2
	Normal control	(1 rat)		6.5
15	1	29	1	12.3
16	1	29	1	10.8
17	½	29	1	11.2
18	½	29	1	11.7
	Castrate control	(Av. 2 rats)		11.6
	Normal control	(Av. 3 rats)		7.6
3	1	29	224	13.2
7	1	29	224	16.6
9	½	29	224	16.7
10	½	29	224	15.4
11	½	29	224	16.7
	Castrate control	(Av. 2 rats)		15.0
	Normal control	(1 rat)		7.6

days (4 rats) and for 21 days (4 rats). Of these animals 1 in each of the 2 groups received a daily dose of ½, 1, 2, or 3 mg. of progesterone. Of 6 males in which treatment was started 1 day after castration, 2 were injected daily for 11 days with 2 or 3 mg. of progesterone, respectively, and 4 were injected daily for 29 days. Two of the latter 4 rats received ½ mg. daily, and 2 received 1 mg. daily. In 5 male rats injections of progesterone were instituted 224 days following gonadectomy. Two of these animals were given 1 mg. daily for 29 days and 3 were given ½ mg. daily for 29 days.

Seven pairs of parabiotic rats with their littermate controls were studied. Four of the pairs were ♂ + ♂ combinations, 2 were ♀ + ♂, and 1 was ♀ + ♀. In each instance 1 partner was completely hypophysectomized before any treatment was be-

gun, and changes in the genital system of this twin reflected modifications in the output of gonadotropic hormone from the pituitary gland of the non-hypophysectomized member of the pair. Use of the hypophysectomized parabiotic rat for this purpose is based upon studies which have been fully described (12, 13). In those experiments it was established that gonadotropic activity of the pituitary gland of a parabiont is suppressed if *a*) in its hypophysectomized male twin the testes and scrotum undergo atrophy, or *b*) in its hypophysectomized female twin vaginal estrus (14) is interrupted for more than a day.

All organs were weighed in the fresh state and fixed immediately afterwards. Hypophyses were fixed in Zenker-formol, cut at 5μ and stained with Mallory's triple stain. Other tissues were fixed in Bouin's fluid, cut at 8μ and stained with Delafield's hematoxylin and eosin.

RESULTS

Experiments on non-parabiotic rats. In the doses given, progesterone failed to prevent or correct castration changes in the pituitary glands of gonadectomized male or female rats. Castration cells and a large number of basophiles were strikingly apparent in the pituitaries of all male and female rats treated with progesterone. No distinct changes were observed in the eosinophiles. Since no cell counts were made, it is not possible to say whether numerical differences existed between the glands of castrated animals which had received progesterone and those of castrated controls. There is no doubt, however, that the hypophyses of the injected castrates were typical of those of gonadectomized animals.

No definite statement can be made concerning the weights of the pituitary glands of the female rats. The number of animals is small and the variations in hypophyseal weights are wide. On the other hand, significant increases in weight seem to have occurred in the hypophyses of male castrates which received progesterone immediately after gonadectomy, as well as in the glands of control castrates. No appreciable reduction in pituitary weight appears to have been brought about by progesterone in castrates in which treatment was begun 224 days after removal of the testes.

Experiments on parabiotic rats.[3] *No. 1.* The pair consisted of N♂ + N♂. The left twin was hypophysectomized, and 21 days later, after its testes had atrophied, the right partner was castrated, making the pair H♂ + C♂. Starting at the time of castration the C♂ received daily for 4 days 2 mg. of progesterone in 0.2 cc. of peanut oil, followed by 39 daily injections of 6 mg. of progesterone in 0.6 cc. oil. One day after the last injection the pair was killed. Weights at autopsy were as follows:

	Testes (*gm.*)	Seminal vesicles (*gm.*)	Pituitary (*gm.*)
H♂	3.09	2.080	——
C♂	——	0.097	0.013
NC♂	4.56	1.120	0.009

The testes and accessory sexual organs of H♂ were histologically normal in appearance. In C♂ the seminal vesicles were somewhat stimulated, indicating an androgenic effect of progesterone, and the anterior pituitary was typically castrate in microscopic appearance, as well as in weight.

From results of this experiment it was concluded that 6 mg. of progesterone injected daily for 39 days into a castrated parabiotic rat failed to suppress the gonadotropic hyperactivity of the anterior hypophysis which occurs after gonadectomy.

[3] Throughout the descriptions to follow, C = castrate, N = normal, H = hypophysectomized, S = spayed, CC = castrate control, and NC = normal control.

This was demonstrated by the fact that the atrophic sexual organs of H♂ were restored to a normal condition by gonadotropin originating from the anterior hypophysis of the castrated parabiont despite the injections of progesterone.

No. 2. The pair consisted of N♂+N♂. One twin was hypophysectomized and 21 days later its testes and scrotum had noticeably retrogressed. The other partner was castrated, making the pair H♂+C♂. On the day following castration and for 43 days thereafter, C♂ received 4 mg. of progesterone in 0.4 cc. peanut oil. Autopsy was performed 1 day after the last injection.

	Testes (gm.)	Seminal vesicles (gm.)	Pituitary (gm.)
H♂	4.08	2.334	———
C♂	———	0.072	0.013
NC♂	4.56	1.120	0.009

As in the preceding experiment, the testes and accessory glands of H♂ were restored from an atrophic state to a completely normal condition, as evidenced by the weights and histological aspect of the organs. In C♂ progesterone was slightly androgenic in its action on the seminal vesicles and prostate, but it failed to prevent hypertrophy of the hypophysis and the appearance of castration cells in the anterior pituitary.

Four mg. of progesterone injected daily for 44 days were therefore incapable of preventing gonadotropic hyperactivity of the castrated rat as judged by the genital response in the H♂ twin.

No. 3. The pair consisted of N♂+N♂. One twin was hypophysectomized, making the pair H♂+N♂. Three months later the testes and scrotum of H♂ were markedly atrophic. Nineteen daily injections of 6 mg. of progesterone in 0.6 cc. peanut oil were administered to N♂. After this series of injections the right testis (weighing 0.41 gm.) was removed from H♂, and N♂ was castrated (its 2 testes weighed 1.28 gm., indicating atrophy caused presumably by progesterone). The pair now was H♂+C♂. Injections of progesterone (6 mg. daily) were continued in C♂ for 24 days. During this period the genital organs of H♂ showed no signs of activity. The injections of progesterone were stopped for 4 days, and in this interval the scrotum of H♂ became turgescent and the testes were easily palpable. Progesterone injections (6 mg. daily) were continued for 7 days, with the result that the genitalia of H♂ became atrophic again. Autopsy was performed 1 day after the last injection.

	Testes (gm.)	Seminal vesicles (gm.)	Prostate (gm.)	Pituitary (gm.)
H♂	1.16[1]	0.051	0.018	———
C♂	———	0.175	0.220	0.010
NC♂	3.00	1.410	0.611	0.012

[1] Doubled weight of left testis. The right testis had been removed previously; its doubled weight was 0.82 gm.

The testis removed at biopsy from H♂ revealed histologically atrophic tubules and interstitial cells. The testis obtained at autopsy was larger than the former, and microscopically it exhibited slight tubular and interstitial cell stimulation. Some of the tubules contained a few spermatozoa. There was histological evidence of slight stimulation of the prostate but none of the seminal vesicles, indicating very slight interstitial cell activation. On the other hand, the prostate of C♂ responded with distinct growth and activity to progesterone, as, to a lesser extent, did the seminal vesicles. Hypertrophy of the pituitary of the C♂ was largely arrested, and castration changes were only slightly evident histologically.

It would seem that 6 mg. of progesterone daily were to a large extent effective in preventing gonadotropic hyperactivity of the castrate pituitary. In all probability the injections given before castration helped initially to reduce gonadotropic activity, as indicated by the decreased size of the testes removed from N ♂.

No. 4. The pair consisted of N ♂ + N ♂. One twin was hypophysectomized, making the pair H ♂ + N ♂. Atrophy of the testes and scrotum in H ♂ was not complete 47 days after removal of the pituitary, indicating that some gonadotropin from the pituitary of N ♂ was reaching H ♂. Injections of progesterone (4 mg. in 0.4 cc. daily) were begun in N ♂ and continued for 23 days. These injections must have depressed gonadotropic function in N ♂, for at castration the testes were found to weigh 1.78 gm. Histologically these testes showed much tubular and interstitial cell damage. One testis removed from the H ♂ at the same time weighed 0.76 gm. The size of this testis and the appearance of the scrotum indicated that the pituitary of N ♂, despite the injections of progesterone, was releasing enough gonadotropin to elicit an appreciable response in the tubules and interstitial cells of the testis. That both testicular elements were stimulated was verified microscopically. Following castration of N ♂, progesterone (4 mg. daily) was administered to the animal for 33 days, at the end of which period the pair was killed. The autopsy weights of the parabionts and of their littermate controls follow.

	Testes (gm.)	Seminal vesicles (gm.)	Prostate (gm.)	Pituitary (gm.)
H ♂	2.92[1]	1.610	0.292	———
C ♂	———	0.107	0.098	0.014
CC ♂	———	0.118	0.028	0.015
NC ♂	3.15	1.104	0.371	0.010
NC ♂	4.35	1.113	0.396	0.010

[1] Doubled weight of left testis. The right testis had been removed previously; its doubled weight was 1.52 gm.

The testis, seminal vesicles and prostate of H ♂ were histologically indistinguishable from normal. Even though progesterone was slightly androgenic in C ♂, hypertrophy and castration changes in the pituitary gland were not prevented.

From results of this experiment it would appear that 4 mg. of progesterone daily depressed hypophyseal gonadotropic activity only to a slight degree.

No. 5. The pair consisted of H ♂ + S ♀. The testes and scrotum of H ♂ were quite normal. Progesterone (1 mg. in 0.5 cc. oil) administered daily for 22 days to S ♀ resulted in no detectable change in the genitalia of H ♂. Atrophy of the testes and scrotum of H ♂ was, however, induced to occur by injections of testosterone propionate (13) in S ♀ (7 injections of 2 mg. each were spread over a month). When progesterone (1–3 mg. daily for 8 days) was substituted for testosterone propionate, renewed activity began in the testes of H ♂. Atrophy of the testes and scrotum in H ♂ recurred upon injection of S ♀ with testosterone propionate. Progesterone (3 mg. daily for 16 days) was again substituted for testosterone propionate in S ♀. This time it proved effective

	Testes (gm.)	Prostate (gm.)	Pituitary (gm.)
H ♂	2.26[1]	0.400	———
S ♀	———	———	0.012
NC ♂	2.32	0.307	———
NC ♀	———	———	0.012

[1] Doubled weight of left testis. The right testis had been removed previously; its doubled weight was 0.84 gm.

in suppressing gonadotropic activity of the pituitary gland of S ♀, for the genitalia of H ♂ remained markedly degenerated. One testis removed at this time weighed 0.42 gm. Following a 17-day rest, the pair was autopsied.

The increase in testicular weight which occurred in H ♂ after treatment of S ♀ with progesterone was discontinued is striking. It should be emphasized that the testis removed at biopsy exhibited complete tubular and interstitial cell atrophy, while that taken at autopsy had normal germinal and interstitial elements. The prostate in H ♂ also was normal. The weight of the pituitary of S ♀ was in the normal range, but it is to be remembered that testosterone propionate may have played a large part in preventing hypertrophy. Castration changes in the anterior lobe were apparent, but not pronounced. Again, this in part was certainly a result of the injections of testosterone propionate.

It is concluded that 3 mg. of progesterone daily were effective in maintaining the inhibition of hypophyseal gonadotropic activity initially brought about by testosterone propionate.

No. 6. After parabiosis of N ♀ +N ♂, the N ♀ was hypophysectomized and the N ♂ was castrated, making the pair H ♀ +C ♂. The vaginal smear of H ♀ soon became cornified and remained so for more than a month. The C ♂ then received progesterone (3 mg. in 0.3 cc. daily) for 36 days. No change occurred in the vaginal smear of H ♀, and the pair was autopsied.

	Ovaries (gm.)	Seminal vesicles (gm.)	Pituitary (gm.)
H ♀	0.365	———	———
C ♂	———	0.017	0.012
CC ♂	———	0.038	0.011
NC ♀	0.070	———	———
NC ♂	———	1.250	0.008

The great enlargement of the ovaries of H ♀ was due chiefly to formation of large follicles, although there was evidence of luteal stimulation. There was pronounced hypertrophy of the pituitary of C ♂, and castration changes in the anterior lobe were markedly advanced.

Progesterone at a daily dose level of 3 mg. did not prevent hypertrophy or gonadotropic hyperactivity of the pituitary gland of C ♂.

No. 7. The pair consisted of N ♀ +N ♀. One twin was hypophysectomized, making the pair H ♀ +N ♀. The vaginal smear of H ♀ became diestrous and remained so for a month. N ♀ was spayed at this time, making the pair H ♀ +S ♀. The S ♀ then received the following consecutive injections of progesterone: ½ mg. in 0.3 cc. oil daily for 3 days, 1 mg. for 1 day, 2 mg. daily for 3 days, and 5 mg. daily for 3 days. Two days after gonadectomy the vagina of S ♀ became cornified and remained so throughout the course of injections. Injections were discontinued, and as the pair died unexpectedly some time later, no organ weights were recorded.

Progesterone at the dose levels used was unable to prevent gonadotropic hyperactivity in S ♀.

DISCUSSION

Under the conditions of the experiments on non-parabiotic rats it would appear that doses of progesterone as high as 3 mg. daily failed to prevent changes in the anterior pituitary which characteristically occur after castration. This finding is at variance with that of Brooksby (4) and of Herlant (15). The amount of progesterone adequate to prevent castration changes in the anterior pituitary was found by Brooksby to be ½ mg. daily and by Herlant, 0.6 mg. daily. Further investigation of this problem seems necessary.

Two conclusions may be drawn from results in parabiotic experiments. *a*) Progesterone was a weak depressant of gonadotropic activity of the pituitary gland. *b*) In hypophysectomized male twins of parabionts injected with effective doses of progesterone, atrophy of the tubular and interstitial elements of the testis always occurred simultaneously and to the same degree. In this respect progesterone would seem to differ only quantitatively from androgen and estrogen (13).

The inability of progesterone to inhibit gonadotropic activity of castrated parabionts sufficiently to terminate vaginal estrus in their hypophysectomized twins may signify that the dose was too low or that only the luteinizing principle of the pituitary was held in abeyance while the follicle-stimulating hormone remained unaffected (11). Considering the high doses of progesterone required to inhibit gonadotropic activity in other parabiotic experiments, it seems more logical to attribute ineffectiveness of progesterone to quantitative insufficiency rather than to its selective inhibition of a gonadotropic factor. Moreover, recent studies on gonadotropic and gonadal physiology (11, 16–20) make it difficult to discuss gonadal stimulation in terms of the conventional FSH and LH.

SUMMARY AND CONCLUSIONS

Progesterone was injected at daily dose levels of ½ to 3 mg. in castrated adult male and female rats starting immediately after or at a delayed period after castration. In no instance did such injections prevent or correct histological castration changes in the anterior pituitary. A distinct hypertrophy of the pituitary occurred in treated as well as untreated castrated males. Weight changes in the glands of female rats were irregular.

The effect of progesterone on the pituitary gland was also studied in parabiotic rats by its ability to influence gonadotropic activity of a castrated parabiont as judged by the testicular or ovarian response in a hypophysectomized twin. Progesterone was found to be a weak gonadotropic depressant, the lowest effective dose being 3 mg. daily.

In instances where progesterone suppressed gonadotropic activity of the pituitary it caused inhibition of the gonadotropin(s) responsible for stimulation of tubular and interstitial elements of the testis. Modifications of the two testicular components were always simultaneous and equal, suggesting that if more than one hypophyseal gonadotropin was involved, progesterone did not exert a selective inhibitory action.

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REFERENCES

1. HOHLWEG, W.: *Klin. Wochnschr.* 14: 1027. 1935.
2. ALBRIEUX, A., W. BUNO, P. ENGLE AND J. MORATO-MANARO: *Klin. Wochnschr.* 15: 206. 1936.
3. FELS, E.: *Compt. rend. Soc. de biol.* 120: 730. 1935.
4. BROOKSBY, J. B.: *Proc. Soc. Exp. Biol. & Med.* 38: 832. 1938.
5. SELYE, H., J. S. L. BROWNE AND J. B. COLLIP: *Proc. Soc. Exp. Biol. & Med.* 34: 472. 1936.
6. PHILLIPS, W. A.: *Am. J. Physiol.* 119: 623. 1937.
7. MAKEPEACE, A. W., G. L. WEINSTEIN AND M. H. FRIEDMAN: *Am. J. Physiol.* 119: 512. 1937.
8. BURROWS, H.: *J. Endocrinology* 1: 417. 1939.
9. HERLANT, M.: *Compt. rend. Soc. de biol.* 131: 1315. 1939.
10. BIDDULPH, C., R. K. MEYER AND L. G. GUMBRECK: *Endocrinology* 26: 280. 1940.
11. ASTWOOD, E. B., AND H. L. FEVOLD: *Am. J. Physiol.* 127: 192. 1939.
12. CUTULY, E., AND E. C. CUTULY: *Proc. Soc. Exp. Biol. & Med.* 37: 477. 1937.
13. CUTULY, E., AND E. C. CUTULY: *Endocrinology* 22: 568. 1938.
14. WITSCHI, E., AND W. T. LEVINE: *Proc. Soc. Exp. Biol. & Med.* 32: 101. 1934.
15. HERLANT, M.: *Compt. rend. Soc. de biol.* 131: 1318. 1939.
16. GREEP, R. O.: *Endocrinology* 23: 154, 1938.
17. ASTWOOD, E. B. *Endocrinology* 28: 309. 1941.
18. CUTULY, E.: *Proc. Soc. Exp. Biol. & Med.* 47: 126. 1941.
19. EVANS, H. M., M. E. SIMPSON, W. R. LYONS AND K. TURPEINEN: *Endocrinology* 28: 933. 1941.
20. SAUNDERS, F. J., AND H. H. COLE: *Endocrinology* 23: 302. 1938.

ANNUAL VARIATION IN THE RESPONSE OF CROP-SACS AND VISCERA OF PIGEONS TO PROLACTIN

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SINCE OUR FIRST PUBLICATION (1) ON THE ASSAY of prolactin by means of the weight of the crop-sacs of doves and pigeons we have studied various factors (2) which modify the extent to which a specific quantity of prolactin increases the weight of the crop-sacs. The present study was undertaken to determine the reproducibility throughout the year of the crop-sac weights obtained at any one time or season. A constant amount of the same preparation of prolactin was used in all tests, and groups of 10 birds were tested at intervals of 4 to 6 weeks during two years. During the first year of study large and unanticipated variations in crop-sac weights were observed at certain periods, and it was therefore decided to continue the study throughout a second year in order to verify the findings of the first year.

The completed study led to the unexpected result that there is a crop-sac cycle of only 6 months' duration, two complete cycles occurring within each year. When crop-sac weights are converted into equivalent units of prolactin the values found during winter and summer (the periods of maximum response) were three times as large as those observed for short periods during spring and autumn. On these same birds we also obtained weights of thyroids, pancreas, testes, liver, heart, intestine and adrenal; length of intestine was likewise recorded. The effect of prolactin on all these values, and the variation of these values with season, are recorded in this report. It is particularly notable that small dosage with prolactin markedly increased the weights of pancreas, liver and intestine, and that the pancreas and liver show some evidence of semi-annual cycles of response similar to those for crop-sacs.

EXPERIMENTAL PROCEDURE

White Carneau pigeons purchased from the Palmetto Pigeon Plant, Sumter, South Carolina, were used. They were shipped to us 6 weeks after hatching (2.0 months after egg was laid); thereafter they were kept in groups in large cages which were maintained at a temperature of 15 to 25° C. in the winter. Injections with prolactin were started 3 days after arrival; recovery from most of the effects of reduced food consumption while in transit was thus permitted before treatment was started. The potent preparation (*no.* 657) of prolactin used was an aqueous insoluble fraction (PH 3.5) which had not been fractionated in 60% ethanol. In comparison with our best preparations of other pituitary hormones its content of gonadotropin was less than 1 part in 1,000; of thyrotropin, less than 1 part in 250; of adrenotropin, 1 part in 20. At intervals of 6 weeks or less groups of 10 pigeons were injected intramuscularly once daily for 4 days with 0.25 cc. of a freshly prepared, aqueous, 1% butanol solution (PH 8) containing 0.5 mg. of *no.* 657 (2 mg. total dosage). The pigeons were killed and the tissues removed and weighed or measured

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on the 5th day. At various times during the 2-year period additional groups of 6 or 7 pigeons were similarly treated with one-fourth the above dosage of *no.* 657 (0.5 mg. total dosage). All statistical deviations are expressed as standard errors.

RESULTS

Crop-sac weight variation. The data are presented graphically in figure 1. The average unstimulated weight of both crop-sacs of a White Carneau pigeon is 1,200 mg. and the maximum stimulated weight obtainable in 4 days is of the order of 7,000 to 8,000 mg. The dosage of prolactin selected for this study was such as would always give crop-sac weights in the middle range of stimulation. The crop-sac weights shown here are not corrected for differences in body weight because any correlation

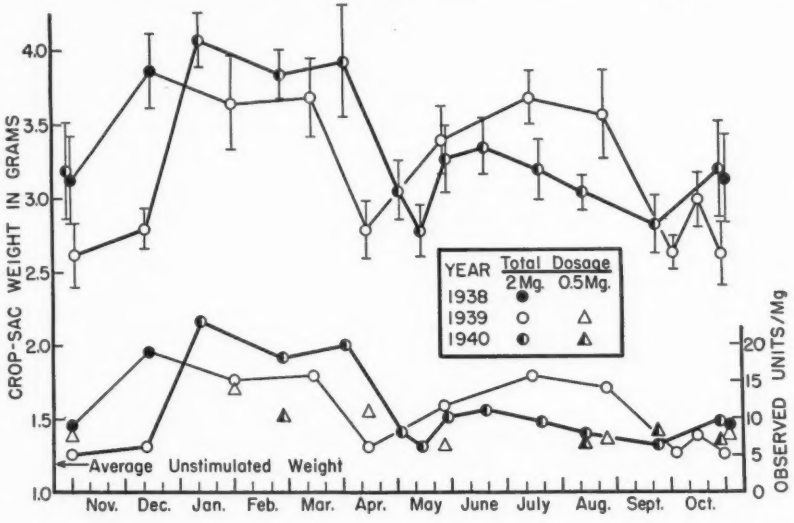

Fig. 1. ANNUAL VARIATION OF CROP-SAC RESPONSE OF 2.3-MONTH-OLD CARNEAU PIGEONS TO *prolactin* 657. Upper curves, crop-sac weights; lower curves, corresponding unitage per mg. of prolactin injected. Each point is the average value for 10 pigeons. Vertical lines through points represent ±.

between the two is of minor importance relative to the seasonal changes. The first group was killed Oct. 31, 1938, and the last group on Oct. 29, 1940. The average crop-sac weights of these two groups were nearly identical thus demonstrating, as did still other tests, the stability of the prolactin powder. The data for the 2 years are superimposed in figure 1 and it is obvious that in general similar results were obtained for the two consecutive years. In each year there were two short periods of minimum responsiveness separated by longer periods of maximum or nearly maximum crop-sac responsiveness.

The fact that a single minimum value was obtained in April 1939 made it necessary to continue the experiment through a second year when an extra group of birds was killed at this period. The curve for the beginning of the second year is seen to be shifted to the right by a time interval of 4 to 6 weeks but it wholly confirms the sudden increase in responsiveness in December–January with return to a minimum in April–May; each change occurred within a period of 4 to 6 weeks. This chronological shift or difference between the two years, 1939 and 1940, is perhaps associated

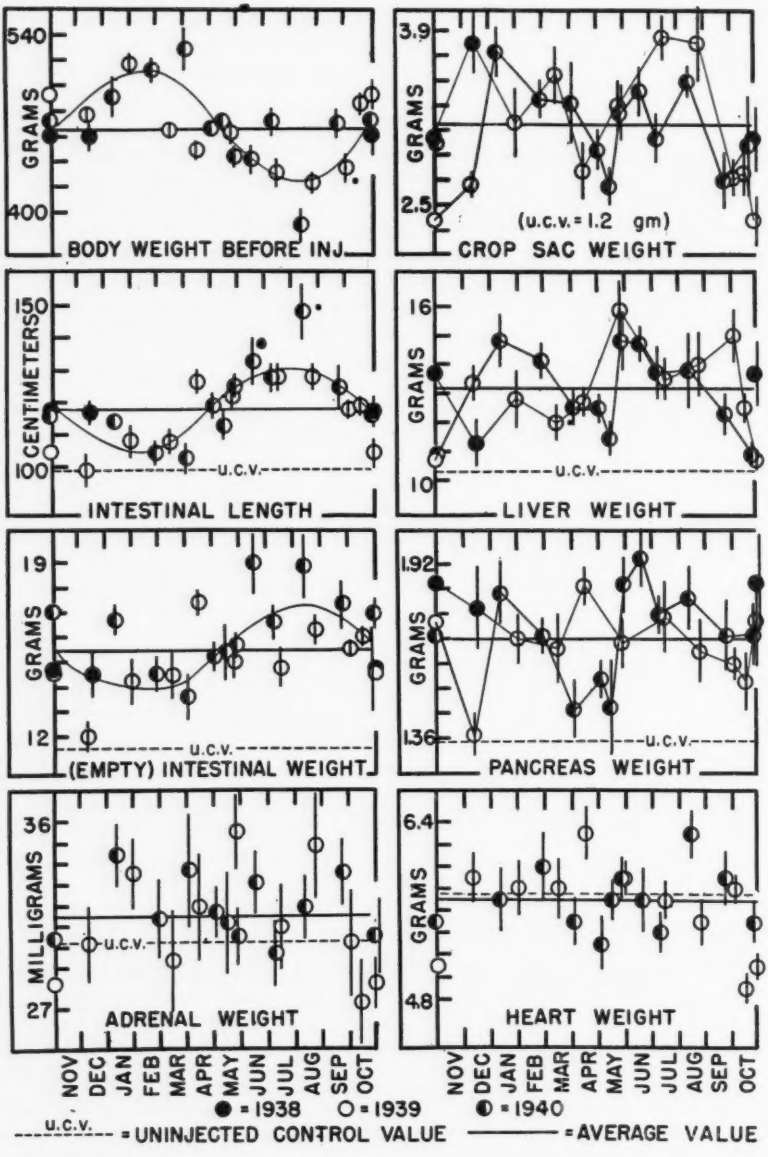

Fig. 2. Seasonal variation in 2.3-month-old Carneau pigeons, prolactin injected. Body wt. (uncorrected), and organ weights corrected to body wt. of 450 gm., of 24 groups of 10 pigeons each. Control values from 70 similar pigeons are indicated.

with the recognized fact that in the Eastern United States cold weather ('late spring') persisted abnormally into the spring of 1940. During neither year was the increase in crop-sac weight in summer as great as that in winter. This difference, however,

is eliminated when the crop-sac weights are corrected to a common body weight since those are the seasons of greatest difference in body weight (fig. 2). The second minimum in September–October was also confirmed by the data obtained at this period during the second year.

The differences in crop-sac weights at the extremes of responsiveness are statistically significant as indicated by the standard deviation of the means shown in figure 1. The minimum average crop-sac weight response (2,617 ± 211 mg.) was obtained on Oct. 31, 1939. The maximum average crop-sac weight response (4,070 ± 182 mg.) was obtained on Jan. 12, 1940. The six groups of high crop-sac weights obtained during the winter averaged 3,837 mg. while the eight lowest values obtained at the two periods of minimum response averaged 2,794 mg. The probability of the difference between these values being significant is greater than 1,000 to 1.

For assay purposes it is necessary to convert crop-sac weights into the equivalent

TABLE 1. COMPARATIVE ASSAY OF PROLACTIN *no.* 657 AND THE INTERNATIONAL STANDARD OF PROLACTIN (I.S.P.) TESTED ON GROUPS OF 10 PIGEONS OF THE SAME SHIPMENT

Date	Preparation	Dosage	Crop-sac wt. σ_m	Units	Relative potency
		mg.	*mg.*		
4/3/40	657	2	3,920 ± 372	48	1.46
	I.S.P.	2	3,558 ± 247	33	1.00
5/3/40	657	2	3,053 ± 201	20	1.43
	I.S.P.	2	2,716 ± 130	14	1.00
7/10/40	657	2	3,189 ± 204	23	0.74
	I.S.P.	2	3,474 ± 345	31	1.00
2/4/41	657	2	3,410 ± 360	29	2.42
	I.S.P.	2	2,575 ± 122	12	1.00

number of units from a chart or table such as that published by Riddle and Bates (3). The lower curves of figure 1 show the results of such a conversion of these data. The unitage values obtained from the smaller dosage of prolactin are indicated by the triangles unconnected by lines; these are of the same magnitude as those obtained with the higher dosage but are too few and scattering to show the cyclic changes also. The plotted values of units/mg. have been corrected for race or strain (White Carneau); that is, after reading off from our published table (3) the number of units corresponding to each group of crop-sac weights that value was divided by 1.2, the racial factor for Carneau. The minimum unitage observed was 5.3 units/mg. and the maximum was 23.3. units/mg. The latter value is 4.4 times larger than the former. Quantitative assays of prolactin must correct for these changes of responsiveness of the crop-sacs of pigeons by parallel tests against a standard preparation of prolactin.

Comparative assay of no. 657 and the International Standard of Prolactin. What should be considered the exact potency of *no.* 657 in view of the above-noted variations? The unitage per mg. as calculated by our method from the average crop-sac weight of all 239 pigeons is 10.7. The geometric means of the unitage from all 24 groups is 10.3. This would indicate an average potency of about 10.5 Riddle-Bates units per mg., which is essentially that of *L.P.* 1, one of the components of the International Standard.

The distribution of the International Standard of prolactin in early 1940 made possible a direct comparison with *no.* 657. At four different times 20 pigeons from the same shipment were divided into 2 groups of 10. One group was injected with

the International Standard and the other with *no.* 657. The results are shown in table 1. The relative potency of the two preparations on April 3 and May 3 (1940) was surprisingly constant although during the short interval of 30 days between these two tests the crop-sac response had decreased by more than 20% in terms of weight and by 55% in terms of units. The two subsequent comparisons, however, were so inconsistent as to question the reliability of results obtained on groups of only 10 Carneau pigeons. The geometric mean of the relative potencies of the 4 comparisons, and the relative potency calculated from the average crop-sac weights of all 40 pigeons on each preparation, were 1.39 and 1.38 respectively. The potency of *no.* 657 is therefore approximately 14 I.U. per mg. This is in agreement with our previous assays (4) of the several original component preparations of the International Standard, all of which (except *L.P.* 1) we found by our technic to contain less than 10 and more than 5.5 (Riddle-Bates) U per mg.; *L.P.* 1 contained 10 such units per mg. Lyons (5) recently reported for *L.P.* 1 a potency of 15 I.U. per mg.

Body-weight. Body weights of pigeons before injection were 20% higher in the winter than in the summer (fig. 2). An annual cyclic change in body weight is indicated by the smooth curve drawn through the average (uncorrected) values for the group of 10 pigeons. These data show that the low response of the crop-sacs in spring and autumn are not ascribable to low body weights of the birds giving those responses. The daily dose of prolactin was too low to cause a marked increase of weight over that of controls. During the 4-day period of treatment the average gain in weight was only 27 gm. per bird; a gain of 5 to 15 gm. is obtained in uninjected birds. Since the body weight of birds differed rather markedly at different times during the year intestinal length and all organ weights were corrected to a common body weight of 450 gm., thus assuming that these values show direct proportionality to body weight. This assumption is probably nearly correct except in the case of intestinal length.

Folley, Dyer and Coward (6), using pigeons of unknown breed and age, and often of very unequal size, found a highly significant correlation between crop-sac weight and body weight; they conclude that unless the body weights of the birds used are kept within rather narrow limits the crop-sac weights must be corrected for body weight. That such a correlation exists and that the correction must be made is obvious when crop-sac weights of pigeons are compared with those of doves of greatly different body weights (1). When the pigeons or doves used in this laboratory were all of a single breed, however, this correction of crop-sac weights for body weight has never seemed to be sufficiently significant to justify its application (2) although we never made statistical calculations to verify this impression.

Consequently for the present material we have calculated the correlation coefficient r for each of the 24 groups of 10 pigeons and for the whole series of 239 pigeons. The value for 239 pigeons was 0.152 ± 0.063 which is low but perhaps significant. The r values for the individual groups were $+0.70$; $+0.69$; $+0.62$; $+0.51$; $+0.47$; $+0.44$; $+0.43$; $+0.42$; $+0.41$; $+0.34$; $+0.31$; $+0.19$; $+0.17$. $+0.06$; $+0.06$ $+0.05$; 0.000; -0.02; -0.05; -0.10; -0.15; -0.25; -0.34; -0.55. In view of the numerous negative correlations the correction of the crop-sac weights for body weight hardly seems justified when the pigeons used are of the same breed and age.

Weight of heart and adrenal. Injections of prolactin did not increase the mean weights of hearts or adrenals over that of uninjected controls (fig. 2, table 2). Mean weights of hearts and adrenals were found to bear a constant relation to body weight throughout the year within the limits of error. When corrected for differences in body weight the weights of adrenals and heart are relatively constant and exhibit no cyclic changes during the year.

Weight and length of intestine. Injections of prolactin significantly increased the

mean length and empty weight of the intestine (table 2, fig. 2). When corrected for differences in body weight the gut length and weight were found to show an annual cyclic variation reciprocally related to the body weight. Actually this apparent annual cyclic variation is due largely to the correction for body weight. In other words, gut length and weight are essentially constant throughout the year and are independent of the changes in body weight. This relationship between gut and body weight is in contrast to that of the heart and adrenal to body weight. Still another type of relationship is found with the liver and pancreas.

TABLE 2. COMPARISON OF MEANS OF MEASUREMENTS OF VARIOUS ORGANS (CORRECTED TO 450 GM. BODY WT.) FROM UNTREATED PIGEONS WITH THOSE OF PIGEONS INJECTED WITH 2 MG. OF *no.* 657. Standard errors of means are given. The significance of the difference between the means is indicted by the significance ratios S.R.)

	Prolactin injected, 239 pigeons	Controls, 70 pigeons	Difference	S.R.
Crop-sac wt., mg.	3170.5 ±50.81	1200.9 ±27.27	1969.6 ±34.16	34.16
Liver wt., gm.	13.25± 0.18	10.29± 0.23	2.96± 0.29	10.21
Pancreas wt., mg.	1689.6 ±21.1	1345.7 ±30.4	343.9 ±37.0	9.29
Gut length, cm.	118.64± 1.14	99.06± 1.55	19.58± 1.93	10.14
Empty gut wt., gm.	15.56± 0.20	11.41± 0.29	4.15± 0.35	11.86
Adrenal wt., mg.	31.54± 0.43	30.18± 0.78	1.36± 0.89	1.53
Heart wt., gm.	5.71± 0.09	5.75± 0.05	0.04± 0.10	0.40

Weight of liver and pancreas. Injections of prolactin significantly increased the mean weight of both pancreas and liver (table 2 and fig. 2). When corrected for body weight the weights of the pancreas, and perhaps of the liver, seem to show a semi-annual cyclic change in weight essentially parallel (in time of occurrence of maxima and minima) to the change in responsiveness of the crop-sacs. The coefficient of correlation between the weights of pancreas and crop-sacs was 0.228 ± 0.062; this is small but statistically significant. The coefficient of correlation between the weights of the liver and crop-sacs was 0.145 ± 0.064; this is small and on the borderline of significance. These semi-annual cyclic changes in responsiveness of, or in the weight of, crop-sac, pancreas and liver contrast sharply with the usual and well known annual (cyclic) seasonal changes. Low dosage of prolactin is here shown to cause rapid and marked overgrowth in the pancreas, liver and intestine of normal pigeons; these data notably support our earlier conclusions concerning the effect of prolactin on these organs of normal (7) and hypophysectomized (8, 9) pigeons.

Weight of thyroids and testes. Weights of thyroids and testes in these young pigeons were not changed by dosage with prolactin. Because of the rapid growth of testes in pigeons of this age, and the further fact that only half of the birds were males, testis variability was too great to permit conclusions regarding seasonal changes in this organ. A 48% increase in thyroid weight of mature doves and pigeons in fall and winter has been described by Riddle and Fisher (10). The present results on young pigeons tend to confirm and extend that report; it is especially notable, however, that a progressive and unexplained annual increase (30–50%) in thyroid weight occurred during a period of 3 years in these young Carneau which exceeds and obscures seasonal change.

DISCUSSION

In considering these results it is essential to recall that all birds used and compared in this study were of exactly the same age at autopsy (7 weeks after hatching; 2.3 mo. from egg). They had learned to eat independently of their parents only 2 to

3 weeks before shipment to us; and while in our hands they were fed precisely the same grain mixture (and grit) which they had learned to eat in the hatchery. Any attempt to interpret the present data involves the consideration of several difficult questions. *a*) Is temperature a controlling factor? *b*) Are the changes observed caused by a seasonal change in responsiveness of the tissues, or by a seasonal change in rate of absorption or elimination of prolactin, whether by excretion or inactivation? *c*) If the second alternative under *b* is true why are the correlations between the weights of crop-sacs and liver and pancreas so small? Why do data for gut weight and length fail to show a semi-annual cycle? Some of these questions can not now be discussed profitably but it is necessary to consider the possible or probable relation of temperature to the cyclic changes in the response of the crop-sacs to prolactin.

Folley, Dyer and Coward (6) have recently shown that temperatures above 15°C. decrease the stimulation of the crop-glands by prolactin. Our only test on the effect of temperature were made on Dec. 11, 1939, when 10 pigeons were kept indoors for our regular seasonal tests at about 20 to 25°C., while from the same shipment of birds another group of 10 pigeons with equivalent body weight and subjected to identical dosage was kept outdoors in a wire pen without protection from the weather during the 4 days of injection. This was a period of frequent high winds, occasional snow and an average temperature close to 0°C. The average crop-sac weight obtained from the group kept indoors was $2{,}795 \pm 135$ mg.; that of the group kept outdoors was $2{,}960 \pm 183$ mg. The difference in crop-sac weights of the two groups is not significant though the apparent difference is in the same direction as that reported by Folley et al. Bearing on this point perhaps is the fact that when corrected for body weight our entire series of 'summer' crop-sac weights are approximately equal to the 'winter' series.

It seems probable that an abrupt transfer of pigeons from ordinary temperatures to a room kept fairly constantly at 30 or 32°C., with prolactin dosage during the following 6 days as this was apparently done in the tests of Folley et al., would cause birds to consume less food than if kept at lower temperatures. This reduced food consumption, even if uncomplicated by the temperature, should reduce the response of the crop-sacs to prolactin (11). Again, it is certain that the lowest metabolic rates, under both basal and non-fasting conditions, are obtained in pigeons measured at 30 to 32°C. (12). It is useful, however, to consider the extent to which changes in food consumption, possibly in association with temperature changes, may have influenced the crop-sac weights obtained in the present series of tests.

Since all our birds were shipped from a warmer or milder climate (South Carolina) to a cooler or colder one (New York) they were required to make some adjustment to temperature in most or practically all cases. In summer this was to a cooler climate. In winter it was into colder weather only during the 24–48 hrs. spent in transit. Thereafter, during a 3-day period allowed for recovery and adjustment and for the 4 following days of treatment, these birds were maintained indoors at a higher temperature than that of the open houses of the hatchery in South Carolina. Though it is true that the heating of our own colony houses begins in October and ends in April or May (the two periods of minimum response to prolactin) the temperature maintained is not high (15–25°C.), and there is evidence that food consumption was little if at all affected at these two periods. Intake of food was not measured directly but it is reflected in the gains in body weight by the birds. Those groups which gave the low crop-sac values of April–May made average gains of 27 gm. per bird, and this is precisely the average gain per bird for the entire series of tests; the birds which gave low crop-sac responses in autumn showed an average gain of 21 gm. per bird, though two of these groups made average gains of 33 and 35 gm. It seems quite

improbable that either temperature, or food-intake as modified by temperature, can provide a satisfactory explanation of the cyclic or seasonal changes in responsiveness.

The seasonal changes which are well known in physiology are annual cyclic changes. Recently Berg, Mayne and Petersen (13) reported that blood pH in humans and dogs is correlated with barometric pressure and perhaps with temperature. We here note that their plotted data show a semi-annual cyclic change which corresponds in time of maxima and minima with our crop-sac data. The coefficient of correlation between the crop-sac weights obtained in this study and the average barometric pressure during the 4-day period of injection was 0.311 ± 0.184; this is not statistically significant, but is perhaps suggestive. There is a possibility that variation in blood pH may account for much of the variation found in these and in still other bioassays.

SUMMARY

In 24 groups of 10 young White Carneau pigeons injected at intervals over a period of 2 years the crop-sacs showed a semi-annual cyclic variation in their weight response to a total dose of 2.0 mg. (28 I.U.) of the same preparation of prolactin.

Two complete cycles of responsiveness were observed in the tests made in each of the 2 years. High values were obtained during winter (highest) and summer. Low values were obtained for much shorter periods during April–May and again during October–November. When crop-sac weights are converted into equivalent units of prolactin the values found during winter and summer are three times as large as those obtained during the short periods of spring and autumn. Season is therefore an important factor in the bioassay of prolactin and it should be controlled by a concurrent test with a standardized prolactin.

The low dosage used in these tests increased body weight over that of the controls by about 4%. Weight of pancreas was increased by 26%; liver by 29%; intestine by 36%. These data strongly reinforce our earlier evidence that in pigeons prolactin has a specific tropic action on these organs.

Weights of testes, thyroids, adrenals and heart were not significantly affected by the treatment with prolactin. Weights of heart and adrenal showed no obvious cyclical or seasonal change.

In these young pigeons the body weights attained at 7 weeks showed an annual variation with a maximum in winter and a minimum in late summer. Gut length and weight at this age were essentially constant throughout the year and independent of different body weights. Corrected to constant body weight these values show annual cycles reciprocally related to body weight. When weights of pancreas and liver are calculated to constant body weight they tend to show a semi-annual cyclic variation similar to that found, with or without that calculation, in the crop-sacs.

REFERENCES

1. Riddle, O., R. W. Bates and S. W. Dykshorn: *Am. J. Physiol.* 105: 191. 1933.
2. Bates, R. W.: *Cold Spring Harbor Symposia Quant. Biol.* 5: 191. 1937.
3. Riddle, O., and R. W. Bates: Sex and Internal Secretions. 2nd ed., Williams and Wilkins, Baltimore, p. 1088. 1939.
4. League of Nations, Bulletin Health Org. 8: 909. 1939. Also, C. W. Emmens, League of Nations, Bulletin Health Org. 8: 901. 1939.
5. Lyons, W. R.: *Endocrinology* 28: 161. 1941.
6. Folley, S. J., F. J. Dyer and K. H. Coward: *J. Endocrinology* 2: 179. 1941.
7. Bates, R. W., O. Riddle, E. L. Lahr and J. P. Schooley: *Am. J. Physiol.* 119: 603. 1937.
8. Schooley, J. P., O. Riddle and R. W. Bates: *Carnegie Year Book* 39: 221. 1939–1940.
9. Schooley, J. P., O. Riddle and R. W. Bates: *Am. J. Anat.* 69: 123. 1941.
10. Riddle, O., and W. S. Fisher: *Am. J. Physiol.* 72: 464. 1925.
11. Bates, R. W., O. Riddle and E. L. Lahr: *Am. J. Physiol.* 127: 422. 1939.
12. Riddle, O., G. C. Smith and F. G. Benedict: *Am. J. Physiol.* 107: 333. 1934.
13. Berg, M., A. Mayne and W. F. Petersen: *Am. J. Physiol.* 130: 9. 1940.

EPIPHYSEAL GROWTH: NORMAL SEQUENCE OF EVENTS AT THE EPIPHYSEAL PLATE[1]

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INTRODUCTION

MOST OF THE LABORATORY WORK on growth has been done by workers using technics such as growth curves, biochemical analyses, and histologic studies of decalcified bone and teeth. In general relatively little roentgenologic research has been done on small laboratory animals. Likewise, limited attention has been devoted to the study of the sites of calcium deposition in histologic material because decalcification is necessary before bones or teeth can be cut. After decalcification it is impossible to identify by usual methods the sites which previously contained deposits of calcium salts.

In this communication new methods for the study of bone are utilized, and attention is devoted to the normal events occurring at the epiphyseal plate of the growing rat as revealed by their application. For a more detailed description of embryology and histology of the bones of the rat the reader is referred to the recent work of McLean and Bloom (1, 2). In subsequent papers studies will be reported of the specific disturbances which follow certain endocrine and dietary experiments. In order to interpret such data, however, it is necessary to visualize the dynamics of normal bone growth since the process can not be adequately understood in terms of descriptive anatomy alone. The tibial epiphysis of the rat has been selected for study because it grows rapidly for several months after birth, because it gives a clear cut shadow on the film and because it resembles the human tibia enough to lend clinical interest to laboratory results. One might call the epiphysis the organ of growth, as it is here that systemic disturbances which affect bone manifest themselves most rapidly.

MATERIALS AND METHODS

The subjects were albino rats reared under good housing conditions on a diet of cereals, minerals, and vitamins in proportions accepted as adequate for normal nutrition.[2] Twenty-three animals were killed at ages varying from 6 to 16 weeks, and the tibiae dissected cleanly at the knee. One bone was fixed in 10% formalin, decalcified and stained with hematoxylin and eosin. The other was stripped of muscular and ligamentous tissue, fixed in 80% alcohol, radiographed and silvered.

Radiography.[3] In the past it has been the custom to expose the whole animal, as for instance on the rat board devised by Shohl (3), using ordinary film. Useful as this may be for

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[1] Aided by a grant from the Proctor Fund for the Study of Chronic Diseases, Harvard Medical School.

[2] 25 lbs. oatmeal, 5 lbs. ground oats, 5 lbs ground meat scraps, 5 lbs. sunflower seed, 5 lbs. whole wheat flour, 5 lbs. powdered milk, 5 lbs. corn meal, 250 cc. cod liver oil.

[3] With the assistance of Oliver E. Merrill, B.E.E., Physicist, Dept. of Roentgenology, Mass. General Hospital.

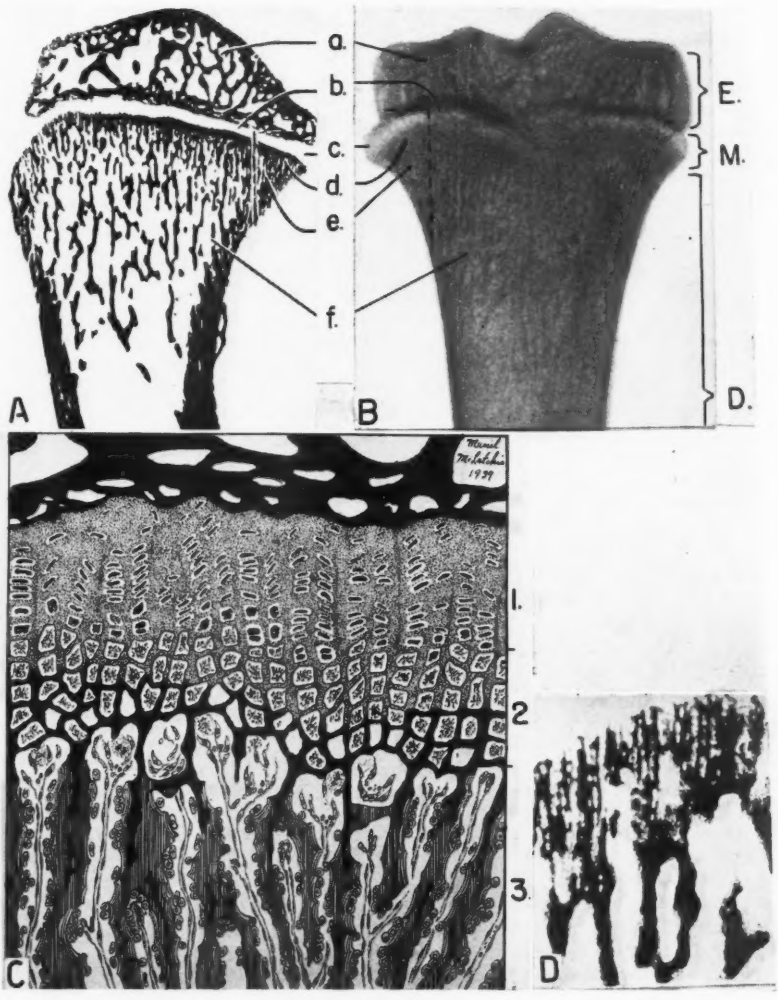

Fig. 1. PREPARATIONS DEMONSTRATING BONE GROWTH IN PROXIMAL END OF TIBIA OF NORMAL GROWING RATS. A, lateral section of proximal end of tibia, Ag impregnation; B, A–P roentgenogram of same region; C, diagram of structure of epiphyseal line and metaphysis: *zone 1* proliferating uncalcified cartilage, *zone 2*, calcified cartilage, *zone 3*, calcified cartilage embedded in newly formed bone; D, Ag preparation of same area.

routine examinations, such a method does not allow for sharp focusing on a given joint and does not give the maximum of detail. More recently O'Brien and Morgareidge (4) have achieved improved detail by holding the animal manually and focusing the tube directly over the knee during a short exposure of dental film. We have found dental film to be more satisfactory than ordinary or non-screen films which have relatively coarse emulsions. We have obtained the best results, most suitable for enlargement and comparison with histologic preparations, by using a fine grain 5 by 7 inches photographic film, panatomic X.[4] The finer

[4] Eastman.

grain of the latter permits greater enlargement without 'graininess' becoming apparent. Our procedure is to kill the animal, and disarticulate the tibia. The fibula and muscular tissue are excised, after which the bone is balanced over the film on its tubercle, a bent paper clip supporting its malleolar end. By elevating the malleolar end of the bone to a proper angle, the epiphyseal plate is brought to lie anteroposteriorly in a plane perpendicular to the film and the shadow cast on the film is consequently clear cut with a sharp hiatus between the center of ossification and the metaphysis representing the uncalcified cartilage (fig. 1, B). The advantages of working with the denuded and disarticulated bone are that the bone may be placed on the film in any desired position and absolute immobility is insured; there is no clouding of the film by muscular and ligamentous tissue, and the length of the exposure becomes the only variable factor.

The only special requirements for the apparatus are an impulse timer for the consistent and accurate control of exposures, and a cone 6 inches long which can be readily constructed from lead sheeting in such a way that its aperture is 1¼ by 1½ inches. The cone protects the person manipulating the bones, and confines the exposure to one small rectangular area of the film. If the tube has no shield or filter, the light from the filament may be used to center the tube over the desired rectangle of the film, successive portions of which may be subsequently exposed. A rotating target tube gives best detail because of small focal spot size.

Exposure factors for non-screen film are 36–40 KV 400 MA 1/20 sec. at 16 inches distance. The panatomic X film requires more exposure: 42–48 KV 100 MA 1 sec. 16 inch distance or 52–60 KV 500 MA 1/10 sec. 16 inch distance. The variation in KV as shown is to cover the size of the various bones encountered. The tibia of a small rat naturally requires less exposure than that of a large rat.

An alternative procedure which occasionally gives striking results is to impregnate the bone with silver as described below and, without sectioning it, to make roentgenograms using exposures of approximately 4 KV more than those recommended above. Because silver absorbs more roentgen rays than do the minerals which compose normal bone, the resulting shadow has increased contrast which is of particular advantage in the study of osteoporotic or rachitic processes.

Histology.[5] Silvered sections of the bones for comparison with and analysis of their roentgenographic shadows are prepared fundamentally according to Gomorri's method (5) which may be summarized as follows: after fixation in 80% alcohol the specimen is washed in water and impregnated with 1.5% silver nitrate solution for 6 to 10 days. After thorough washing it is placed in a reducing solution of 5% sodium hypophosphite for 4 to 8 days. It is washed again; fixed in a 3 to 5% solution of sodium thiosulphate for 2 days; rewashed; and then decalcified in a 6 to 8% solution of sulphosalicylic acid. It is then embedded in paraffin and sectioned on the microtome.

According to Gomorri when the bone is immersed in silver nitrate the following chemical reactions occur: "As silver phosphate is much less soluble than calcium phosphate, and silver carbonate also considerably less soluble than calcium carbonate . . . calcium will be supplanted by silver, at least in the phosphate part, in an inverse proportion to the solubility of calcium and silver respectively." By reducing the silver phosphate thus obtained to metallic silver and removing any calcium salts that have not partaken of the reaction by 4% sulphosalicylic acid, black metallic silver is left at the site of the lime salts.

Modification. Gomorri advocated the use of a dark room to prevent light from reducing the silver salts to metallic silver. However, we have found that the various steps can be carried out more conveniently in small jars rendered light proof by several coats of black enamel. They are fitted with two-holed rubber stoppers; a short piece of glass tubing is inserted into one of the holes, and a longer piece reaching to the bottom of the jar into the other. This latter piece of tubing can be advantageously bent to allow for more space in the interior and at the same time its end can be drawn out to a bore which will be small enough to allow the formation of small bubbles when air is forced through it, and large enough to allow passage of a small stream of water.

With such jars solutions can be readily sucked out and replaced, and continuous agitation effected by a stream of air bubbling through the longer tube. This greatly facilitates penetra-

[5] With the assistance of Thomas J. Blake, Mosher Laboratory, Massachusetts Eye and Ear Infirmary.

tion of the dissolved chemicals and if the bone is sawed in half at the mid-shaft (so that the marrow cavity is opened to internal penetration by the solutions) permits the use of the whole epiphysis instead of thin sections as advocated by Gomorri. Continuous lavage of the bone is insured by suspending it by a thread clear of the bottom, so that it rocks freely the whole time that it is immersed.

After decalcification and cutting, the sections can be counter-stained with the usual dyes, but histologic 'silhouettes' such as that illustrated in figure 1, A, are mounted just as they come from the microtome.

RESULTS

In figure 1, a silvered section of the tibia of a normal rat, A, is compared with a fine grain roentgenogram of the same bone, B. The section is cut in a lateral plane while the roentgenogram is an antero-posterior view. Their contours differ in that there is no flare at the epiphysis in the region of the anterior tibial tubercle of the section.

Roentgenographically the bone may be easily subdivided into the epiphysis, E; metaphysis, M; and diaphysis, D (fig. 1, B). Histologically the epiphysis is seen to include the center of ossification, *a*; the proliferating cartilage, *b*; and the cartilage which composes the chondrophyte, *c*. The two latter structures being uncalcified cast no shadow on the film and are unaffected by silver impregnation. The metaphysis includes the calcified cartilage, *d*, and its bony encasement, the primary spongiosa of the histologist. For that part of it which flares beyond the natural curve of the cortex to form an out-cropping supporting the chondrophyte we suggest the name metaphyseal buttress, *e*. The metaphysis is the zone of active endochondral bone formation, a 'barometer zone' as it were, reflecting the earliest changes in the biochemical milieu which affect the growth pattern. It merges imperceptibly into the secondary spongiosa, *f* (fig. 1, A). The buttress is a very important area roentgenologically speaking, since being peripherally located, it can be visualized in clearest delineation and since being part of the zone of endochondral bone formation it is one of the first areas to show pathologic change.

Normal Growth

Outline of Events: A diagram of the epiphyseal plate (fig. 1, C) and a brief outline of events which are known to be occurring there will clarify the development of our material.

The growth of bone is the result of several independent, though integrated, processes involving:

(*a*) Growth of new cartilage from the germinal cells just under the bony plate of the center of ossification (*zone 1*). The epiphysis is thus moved away from the metaphysis.

(*b*) Senescence and degeneration of the oldest cartilage cells that is those nearest the metaphysis, with precipitation of lime salts in their matrix. This rigid base, the so-called calcified cartilage (*zone 2*), forms a support for the weight-bearing epiphysis and distributes the load over the diaphysis.

(*c*) Erosion of the calcified cartilage into a honey-comb of cell spaces by capillary tufts and osteoclasts advancing from the marrow.

(*d*) The incorporation of most of the calcified cartilage directly into subjacent bone trabeculae (primary spongiosa *zone 3*) by osteoblasts; and coincident resorption of the rest by osteoclastic activity.

(*e*) Continuous revision and remodeling of the submetaphysis and cortex. As the bone enlarges certain trabeculae which were formerly in stress-bearing alignments, are no longer so situated .They are resorbed by osteoclastic activity and replaced by hematopoietic marrow while new trabeculae are formed to meet the new stresses.

Likewise, the cortex is excavated internally and augmented externally by the periosteal osteoblasts.

The aggregate result of these processes is continuous growth. It is a steady lifting of the epiphysis by the proliferating cartilage cells like the hoisting of a huge platform by the combined efforts of a large number of laborers. A temporary scaffolding as it were, holds the platform until a permanent superstructure can be constructed.

Roentgenologic and Histologic Details: It must be borne in mind that in the roentgenogram or microscopic section of a growing bone we view the totality of the several processes suspended as they were occurring when the exposure or section was made. Viewed either roentgenographically or under low power magnification endochondral growth is an extremely orderly phenomenon. The continuously growing cartilage is of uniform width, and is not calcified; though from its metaphyseal surface calcified trabeculae arise with almost military precision to form a vertical scaffolding which through a series of gothic arches provide a firm support for the weight-bearing

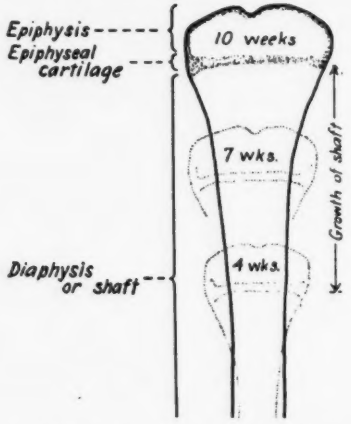

Fig. 2. Reproduction of illustration of bone growth in rat, Dodds and Cameron.

epiphysis. These trabeculae are most numerous and delicate at the metaphysis, and become coarser and less numerous in the submetaphysis (secondary spongiosa). Eventually they merge into the cortex, so that the diaphysis is practically a hollow cylinder. The silvered section (fig. 1, D) illustrates in a single plane these trabeculae which in the roentgenogram are obscured by the superimposition of numerous other planes which go to make up the whole bone.

The cortex of the growing bone which is of very considerable thickness in its diaphyseal portion gradually tapers towards the metaphysis where it disappears altogether in a V-shaped outcropping which forms a support for the chondrophyte. This we have called the metaphyseal buttress. It is composed of fine longitudinally-placed trabeculae originating in the calcified cartilage and ending in the periosteum (fig. 3 E, F), and its extent varies considerably with the gross contour of the bone (fig. 1, A, B). Since the contour of bone remains unchanged while elongation takes place, there must occur first the formation and subsequently the removal of this V-shaped portion which flares out past the natural cortical curve. This process is called tubulization. To quote Dodds and Cameron (6) whose illustration based upon studies of the rat we have reproduced here (fig. 2): "The need for this removal be-

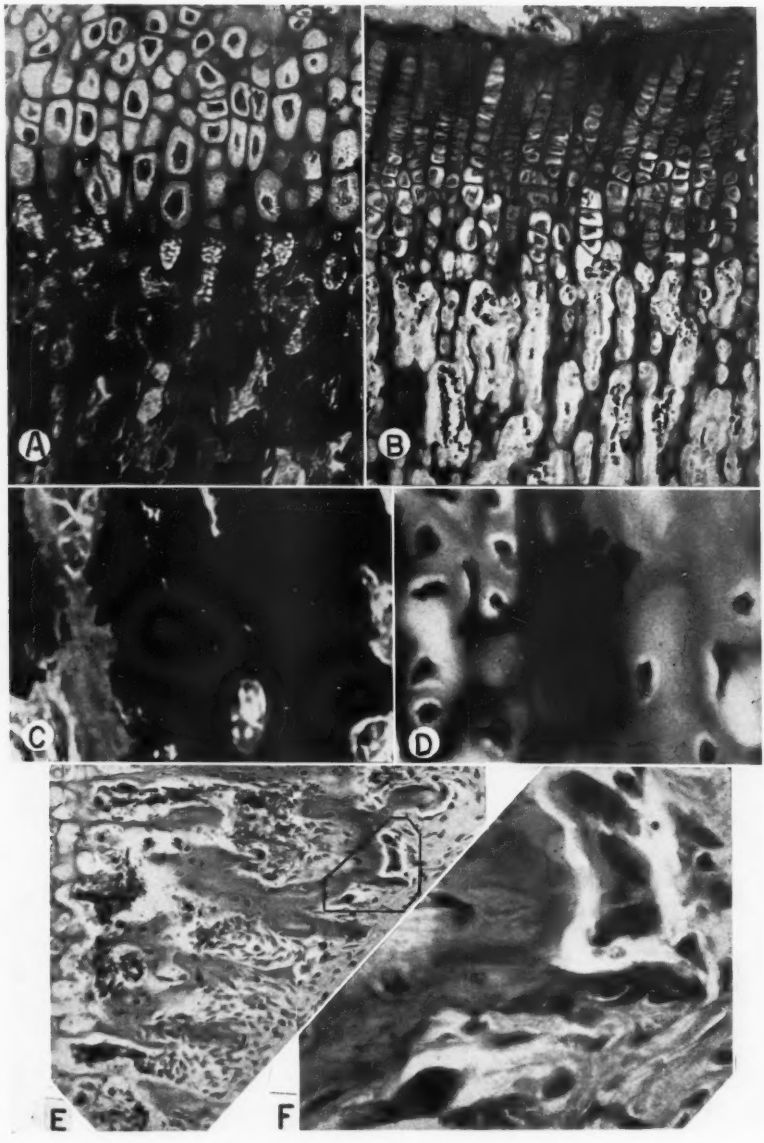

Fig. 3. Bone growth in normal growing rat. *A*, Ag preparation of junction of cartilage and shaft of rat tibia, counterstained H. and E.; bone and calcified cartilage stain black, indicating the extent of Ca deposition in the matrix of degenerating cartilage cells. Capillaries can be traced into the degenerating cartilage cells osteoblasts may be seen about bone trabeculae. ×216. *B*, comparable area through epiphyseal plate, H. and E. Capillary loops are visible, and cartilage can be distinguished from bone. There is no way to identify the extent of mineralization in this preparation. ×144. *C*, Ag preparation of primary spongiosa. Osteoid laid down by osteoblasts indistinguishable from preëxisting bone and calcified

(*Legend continued on next page*)

comes evident when we superpose x-ray photographs or scale drawings showing successive stages of a growing bone." Likewise, the development of the shaft involves not only an increase in length but also an increase in width. This is clearly illustrated by the same diagram. The cortex is steadily being augmented externally by the osteoblasts of the periosteum, while internally, gradual excavation and enlargement of the bore is effected by osteoclasts.

With high power magnification the following details may be observed in the histologic preparation (fig. 3 A, B). Beneath the bony plate of the center of ossification

Fig. 4. SECTION OF JUNCTION OF CARTILAGE AND SHAFT OF TIBIA OF GROWING RAT showing capillary penetration and encasement of cartilage matrix by bone and osteoblasts, H. and E. Note poor staining of 2 cartilage cells in left center. ×408.

lie the germinal cartilage cells, proliferation of which toward the shaft pushes the epiphysis away from it. These cells maturate in parallel columns (columnar cartilage) and eventually become relatively large so that the extent of their intercellular matrix becomes correspondingly reduced. The oldest cells, those farthest from the germinal layer, show degenerative changes, first, pyknosis and then karyolysis (fig. 4). The cytoplasm also stains less distinctly and sections prepared by the Gomorri technic reveal the deposition of calcium salts in the intervening matrix (fig. 3, A). The cell spaces adjoining the marrow cavity are found to contain capillary loops and accompanying osteoblasts advancing from the marrow. Crescents of these cells line the lacunae and appear to phagocytize the cellular debris; presumably they, or the capillaries, are the active agents in the dissolution of the septa of calcified cartilage. Transverse septa are for the most part eroded as the capillaries penetrate this region, while

cartilage. ×356. *D*, Comparable section to C, H. and E.; calcified cartilage can be distinguished from bone. Note that whole sections of intact cartilage are incorporated in bone and many lacunae formerly occupied by cartilage cells contain osteocytes; *E*, Section of buttress of tibia. Trabeculae appear to arise from cartilage as they do elsewhere along epiphyseal line. ×72. *F*, Enlargement of area indicated in *E* showing large osteoclasts at surface of resorbing bone.

most of the longitudinal septa are incorporated directly into bone trabeculae. In many locations, however, whole sections of intact cartilage matrix are embedded en masse in the bone by osteoblasts which invade lacunae formerly occupied by cartilage cells and deposit their own matrix, the so-called osteoid (fig. 4, center). Mineralization of osteoid is now the final step resulting in the production of true bone. Deposition of calcium salts in osteoid occurs so rapidly that only a thin seam of uncalcified matrix (estimated by Shipley et al. (7) to be from 1 to 11 mu wide in the rat) can be found around normal bone.

The formation of bone in the peripherally located 'buttress' does not appear to differ in any important respect from the formation of bone elsewhere along the epiphyseal line. The resorption of trabeculae in this area appears to be brought about by large osteoclasts near the periosteum such as those shown in figure 3, F. Evidently resorption keeps pace with production since the symmetry of growing bone remains relatively constant.

The selection of certain spicules of calcified cartilage for incorporation into bone seems to be governed by the forces of stress and strain which are distributed through the series of props and gothic arches throughout the bony structure. Where there is no need for bone, none seems to be formed, and resorption of pre-existing bone or mineralized cartilage by osteoclasts begins to occur in such situations, whether superficial or deep. Conversely, trabeculae are formed in what seems a purposeful manner where they are needed. In general, the main trabeculae are parallel to the long axis of the bone and it is where they abut the mineralized cartilage spicules that the embedding of the latter in bone, like iron bars in reinforced concrete, seems most likely to occur.

The net result of these processes is the production of a shock-absorbing weight-bearing epiphysis capable of growth but securely incorporated into the underlying bony metaphysis.

DISCUSSION

The anatomy of the epiphyseal plate has been reviewed in some detail, since its normal histologic picture forms the basis for analysing abnormal patterns of growth, and studying the factors influencing growth.

The most important of these factors are: *a*, nutritional, involving among other things an adequate supply of Ca, P, vitamin D and vitamin C; *b*, endocrine, including the active principles of the pituitary, thyroid, parathyroid, and sex glands; *c*, toxic, from the ingestion of harmful amounts of lead, phosphorus, bismuth or fluorine. It can be shown that within the space of a few days disturbances of these regulating influences may be registered morphologically at the epiphysis. When the deviation from the normal develops to a pathognomonic degree, the histologist and the roentgenologist can diagnose rickets, scurvy, lead and phosphorous poisoning, cretinism, and the like.

In this study we have used new roentgenologic and histologic methods to investigate the sites of calcium deposition at the cartilage shaft junction. Calcium salts may be demonstrated in both the longitudinal and horizontal septa of the degenerating cartilage cells lying just above the capillaries advancing from the shaft (fig. 3, A). This finding based on calcium stains differs from Dodds and Cameron's interpretation of decalcified sections that calcium salts are deposited in the longitudinal walls between columns of cells but not in the transverse septa. They felt that the transverse septa remained uncalcified and therefore could be more easily removed than the longitudinal walls by the advancing marrow. In this process cartilage cells were "liberated," and the calcification of the longitudinal walls served "the double purpose of

guiding the advancing marrow and of forming a base on which the first bone is laid." They, apparently, believe that calcified matrix is resistant to dissolution and that uncalcified matrix is susceptible to dissolution by osteoclasts.

Our own results lead to the opposite conclusion, namely that the cartilage cells must die and their matrix become calcified before it is stabilized enough to be remodeled by the marrow substance below. First, as already mentioned, we can demonstrate calcium deposits in both the transverse and longitudinal septa. Unless calcium stains are used there are no reliable criteria for differentiating calcified from uncalcified areas. Second, we believe we can demonstrate the death of the cartilage cell rather than its liberation (fig. 4). Our conclusions about this long contended point are in harmony with Wolbach's statements (8): "Calcification takes place coincidentally with the degeneration of the cartilage cells and only in the matrix surrounding cells which show very evident morphological aspects of degeneration," and "death of the cartilage cells is an essential to growth of bone. Not only does it permit the ingrowth of capillaries but it is attended by the deposition of calcium salts in the surrounding matrix."

In connection with ingrowth of the capillaries we wish to emphasize the great number of osteoblasts to be found in the interstices of the calcified cartilage. Their importance as phagocytes has been stressed only by students of the reticulo-endothelial system who have shown their intense phagocytic activity by injections of colloidal dyes (9), carbon (10), and thorium dioxide (11). In many places they appear to fan out gradually from the capillary endothelium as though they were derivatives of it, or its connective tissue investment. Because of this demonstrable phagocytic activity, it seems possible that these cells are the active agents in the dissolution of the septa of calcified cartilage, and that the rôle of the capillary is secondary. That these same cells, or cells morphologically indistinguishable from them, are capable of laying down a pre-osseous matrix soon becoming calcified has seldom been questioned. However, the Blooms (2) have recently stated "that in the early development of rat bones a stage of osteoid tissue is but a minor phenomenon and is not always present." This may be true for embryonic bone (which is largely calcified cartilage) but, at the rapidly growing metaphysis, we have found osteoid matrix very much in evidence (fig. 3, E, F). We believe that Shipley's statement (7) of 20 years ago still sums up the situation of osteoid tissue concisely and accurately: The "calcified substance (of the cartilage) serves as a core about which . . . a preosseous tissue . . . is formed by the osteoblasts. This very rapidly receives a deposit of calcium and becomes true bone. Calcification of the preosseous tissue is very rapid so that only a very narrow border (1 to 11 mu) of osteoid tissue can be found about bone trabeculae in normal young animals. This is known as the physiological osteoid."

In viewing the problems of growth from clinical and roentgenological points of view it is essential that the differentiation of calcified cartilage from bone be kept in mind, together with the conception of how the former grows, calcifies and becomes sunk or embedded in the latter. It is important to appreciate that the roentgenologist is confronted with gross rather than with microscopic changes. Moreover, he has only the shadow cast by the mineralized structures to interpret. Much of the cartilage casts no shadow. In this study, therefore, considerable attention has been placed on roentgenologic details as correlated with the actual sites of calcium deposition in the epiphysis.

Further work is underway using these methods to investigate endocrine and dietary factors controlling epiphyseal development. For the interpretation of abnormal patterns resulting from experimental procedures, it must be continually borne in mind that there are several correlated processes involved in bone growth, and that

disturbances must be analysed in terms of these components: proliferation, degeneration, and calcification of the cartilage; removal of calcified cartilage; capillary penetration; production of osteoid matrix and its calcification or lack of it. Each must be scrutinized in turn.

SUMMARY

In this project new roentgenologic and histologic methods have been applied to a study of endochondral bone formation. Since the epiphyseal cartilage together with the bony metaphysis, situated at the extremities of rapidly growing bones throughout the body constitutes the organ of growth, the anatomy of this region has been dealt with in some detail.

The cartilage is composed of germinal, proliferating, and degenerating layers; the metaphysis is composed of a rind of new bone laid down by osteoblasts around the calcified matrix of degenerated cartilage cells. An important part of the metaphysis is its peripheral outcropping or buttress. In the interstices of the metaphysis are capillaries, osteoblasts, and osteoclasts, the active agents in the development and remodeling of true bone. Calcium depositions were demonstrated in both the longitudinal and transverse septa of the matrix of the degenerating cartilage cells. This finding is at variance with the conclusions of some writers that calcium salts are deposited only in the longitudinal septa, and harmonizes with Wolbach's belief that mineralization of the cartilage matrix is a prerequisite for its orderly invasion and resorption by capillaries and phagocytes. The deposition of calcium salts in the ground substance appears to follow the death of the cartilage cells.

REFERENCES

1. McLean, F. C., and W. Bloom: *Anat. Record* 78: 333. 1940.
2. Bloom, W., and M. A. Bloom: *Anat. Record* 78: 497. 1940.
3. Shohl, A. T.: *Proc. Soc. Exp. Biol. & Med.* 28: 770. 1931.
4. O'Brien, B., and K. Morgareidge: *J. Nutrition* 16: 91. 1938.
5. Gomorri, G.: *Am. J. Path.* 9: 253. 1932.
6. Dodds, G. S., and H. C. Cameron: *Am. J. Anat.* 55: 135. 1934.
7. Shipley, P. G., E. A. Park, E. V. McCollum and N. Simmonds: Dental Cosmos. 1922.
8. Wolbach, S. B.: De La Mar Lecture. Johns Hopkins Hospital. 1937.
9. Shipley, P. G., and C. C. Macklin: *Am. J. Physiol.* 42: 117. 1916.
10. Wislocki, G. B.: *Bull. Johns Hopkins Hosp.* 32: 132. 1921.
11. Higgins, C., and K. Smith: *J. Exper. Med.* 67: 41. 1938.

EPIPHYSEAL GROWTH: THE EFFECT OF REMOVAL OF THE ADRENAL AND PITUITARY GLANDS ON THE EPIPHYSES OF GROWING RATS[1]

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IT IS WELL KNOWN that hypophysectomy is followed by a cessation of growth (1). Freud, Levie, and Kroon (2) who have studied the specific effect of this procedure on the epiphysis found that the defect was "definitely localized in the growing epiphyseal cartilage," and characterized the histologic changes as "exactly analagous to those exhibited at the end of the normal growth period." No one as far as we know has studied the effects of adrenalectomy on the epiphysis.

We have studied the effects of both procedures on the epiphysis using roentgenograms and silvered sections of bone as well as the usual histologic preparations. We have included studies of both glands in the same project since after adrenalectomy we have obtained histologic evidences of cessation of growth which are not unlike those following pituitarectomy.

MATERIALS AND METHODS

Albino rats of both sexes, approximately 100 gm. in weight and 8 weeks of age were employed as experimental subjects. They were maintained on a diet of cereals described in a previous paper (3), which included minerals and vitamins in amounts accepted as adequate for normal nutrition. Thirteen animals were pituitarectomized by a parapharyngeal approach (4) and subsequently killed. Serial sections on the brains of the hypophysectomized animals were not made although gross examination in all cases showed no evidence of residual pituitary. The characteristic appearance, weight curve and sexual immaturity of the animals were all taken as additional evidence of successful hypophysectomy.

A second group of 11 rats were adrenalectomized through a lumbar incision under ether anesthesia. Both glands were removed at the same operation, great care being taken to remove all adrenal tissue including the capsule. The animals were autopsied after varying periods and were then carefully examined for remnants of normal or aberrant adrenal tissue.

A third group of 12 animals served as controls.

One intact tibia was fixed in 10% formalin, decalcified, sectioned, and stained with hematoxylin and eosin and Masson's trichrome stain; the other was fixed in 80% alcohol and then studied roentgenographically by Ingalls' modification of Gomorri's silver stain. Pathological sequences at the epiphyseal plate were then compared with normal events as recorded in a previous paper (3).

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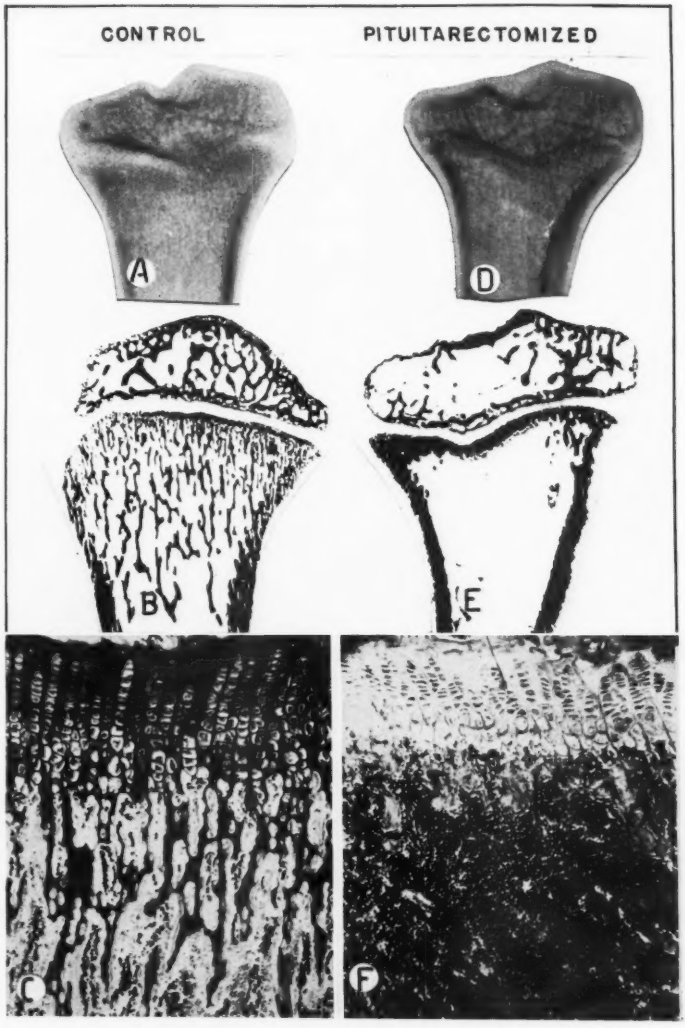

Fig. 1. PREPARATIONS OF PROXIMAL END OF TIBIA OF GROWING RAT. *A*, *B* and *C*, control; *D*, *E* and *F*, pituitarectomized 14 days previously. *A* and *D*, roentgenogram; *B* and *E*, Ag preparation; *C* and *F* microscopic section, H. and E. stain.

RESULTS

Effect of Pituitarectomy. During the first week following pituitary ablation the appearance of the cartilage-shaft junction showed no great deviation from the normal. Between 7 and 13 days there developed a progressively severe atrophy of both the primary and secondary spongiosa which could be demonstrated in the roentgenogram and in the histologic section (fig. 1, D, E).

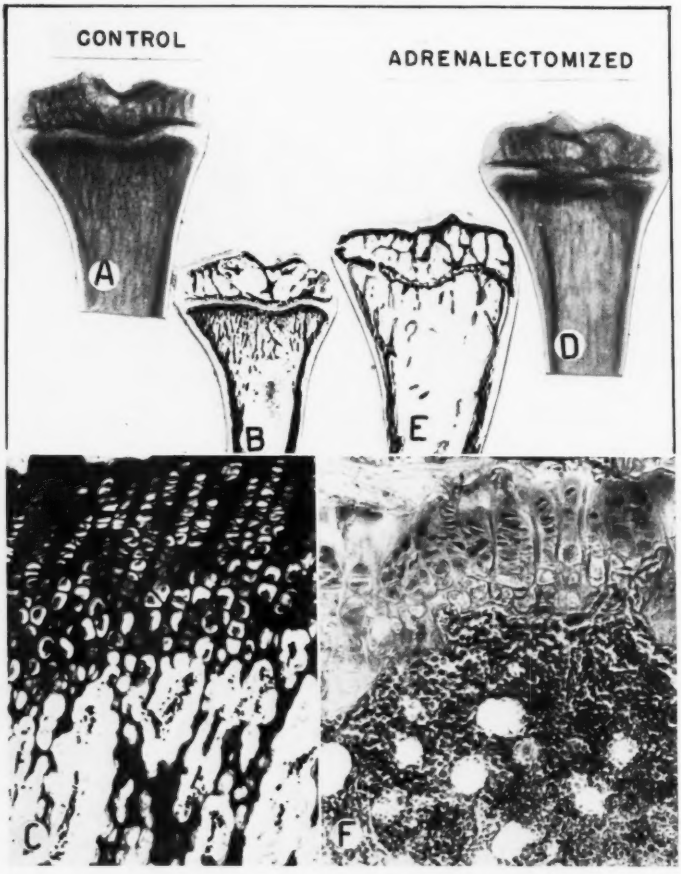

Fig. 2. PREPARATIONS OF PROXIMAL END OF TIBIA OF GROWING RAT. *A*, *B* and C control; *D*, *E* and *F*, adrenalectomized 13 days previously. *A* and *D*, roentgenogram; *B* and *E*, Ag preparation; C and *F*, microscopic section, H. and E. stain.

By the 13th to the 20th day there was a definite diminution in thickness of the epiphyseal cartilage so that its appearance in a rat of 10 to 11 weeks of age was not greatly dissimilar to that found in older animals when the rate of growth has begun to slow (fig. 1, F). There was a relatively abrupt change between the layer of flat germinal cells and the fully grown degenerating cells. There appeared to be an inhibition or lack of proliferating columns. Fully grown cartilage cells instead of being rectangular and elongated were round and stubby. Calcium salts could be demonstrated in the matrix of the cartilage cells at the cartilage-shaft junction. The metaphyseal portion of the calcified matrix, however, instead of being a series of regular, finger-like projections was stunted and clubbed. It was covered for the most part by a thin rind of bone, which seemed to seal off the calcified cartilage from the marrow substance. Fewer osteoblasts and less osteoid than are normally present were seen and in general there appeared to be a fairly complete interruption of metaphyseal

bone formation. There also appeared to be a diminution in the number of capillaries which ordinarily penetrate the lacunae of the calcified cartilage. Occasional capillaries could be seen at the cartilage-shaft junction; but a loop was not found in the degenerated cell at the base of each row as it is normally (fig. 1, C).

Effect of Adrenalectomy. The earliest changes observed after adrenalectomy occurred at 5 to 8 days and consisted of an atrophy or flimsiness of the primary spongiosa and ground substance of the degenerating cells. The matrix was sparsely and poorly calcified. Calcification was not absent as it is in rickets but was merely scanty, fragile in appearance and did not extend up between the cells in an orderly fashion. In the metaphysis the disorder appeared to be characterized by a cessation of osteoid formation and a beginning atrophy of existing bone.

Only 3 animals survived adrenalectomy long enough to exhibit changes of more than 10 days' duration. There was a marked and widespread demineralization of the whole bone which could be demonstrated in the roentgenogram and in the histologic section (fig. 2, D, E). The changes included a marked irregularity and stunting of the columnar cartilage (fig. 2 F). As in the pituitarectomized animals there was an absence of finger-like extensions of calcified matrix, and a thin rind of bone was found on the metaphyseal side of this blunted matrix. Capillaries could be made out in this area but a loop was not found at the base of each row as it is normally. In order to maintain some other animals longer than the 5 to 10 days they usually survive adrenalectomy, 3 rats were given small subcutaneous injections of a cortical preparation[3] for a period of 10 days, then deprived of the extract, and autopsied postoperatively 15, 15, and 17 days respectively. In these 3 animals the findings were duplicated.

The tibiae of 12 control animals showed no abnormalities.

DISCUSSION

In any study of epiphyseal growth it is essential to analyze each of the component processes, proliferation and degeneration of the cartilage; calcification of the ground substance; resorption of the calcified cartilage; capillary penetration, and finally bone formation itself, the production of preosseous matrix by osteoblasts and the deposition of mineral salts within it.

The histologic features in the tibial epiphyses of the rat following both pituitary and adrenal ablation were very similar. There was an absence or inhibition of cartilage proliferation and an absence or inhibition of metaphyseal bone formation. This was accompanied by a definite decrease in the number of osteoblasts and amount of osteoid normally present. A feature demonstrable by roentgenograms and silvered preparations most marked in the adrenalectomized rats was halisteresis of the whole bone.

Our results with pituitarectomized rats confirm Freud, Levie, and Kroon's finding that there is a defect in growth demonstrable at the epiphysis. However we cannot subscribe to their conclusion that the histological features "are exactly analogous to those exhibited at the end of the normal growth period." We believe there is a widespread atrophy of the cartilage and primary spongiosa; and an halisteresis of the whole bone which is distinctly pathological. This is not to deny that there may be an element of suppressed pituitary secretion involved in maturation and closure of the epiphyses. Such a simple explanation does not adequately account for the cyclic nature of epiphyseal closures (5).

A disorder similar to that which follows pituitarectomy is found after adrenalectomy. Only 3 animals survived adrenalectomy long enough to exhibit well-marked

[3] Cortin, Upjohn, Kalamazoo, Michigan.

arrest of growth, but the number of animals surviving long enough to exhibit this finding was increased by the use of small doses of cortical extract parenterally administered.

It is possible that both the disturbances described have a common origin in the cessation of function of the adrenal cortex, and that the disorder of the epiphysis following ablation of the pituitary is to some degree at least dependent upon the atrophy of the adrenal cortex which has been demonstrated to be an inevitable sequence of that procedure.[4]

SUMMARY

A failure of endochondral bone formation similar to that which follows hypophysectomy was demonstrated after ablation of the adrenal glands. However, the departure from the normal and the demineralization of the bone seemed more acute and widespread than was the case for the hypophysectomized animal in the same length of time.

It is evident that the normal functioning of the adrenal gland is as essential for continued growth of the epiphyseal plate of the rat as is the normal functioning of the hypophysis. It is suggested that the atrophy of the adrenal cortex which has been shown to occur after hypophysectomy may be a major factor in the interruption of growth which also follows that procedure.

REFERENCES

1. Evans, H. M.: J.A.M.A. 101: 425. 1933.
2. Freud, J., L. H. Levie and D. B. Kroon: *J. Endocrinology* 1: 56. 1939.
3. Ingalls, T. H.: *Endocrinology* 29: 710. 1941.
4. Thompson, K.: personal communication.
5. Dawson, A. B.: *Anat. Rec.* 31: 1. 1925.

[4] After ablation of the pituitary body a "striking atrophy overtakes the adrenals, and this reduction is due almost entirely to the atrophy of the cortex which becomes little more than a shell" (1).

QUANTITATIVE ASSAY OF THE MELANOPHORE-DISPERSING HORMONE DURING THE DEVELOPMENT OF THE CHICKEN PITUITARY[1]

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TWO OBSERVATIONS SUGGESTED the rather early appearance of the melanophore-dispersing hormone during ontogeny. It was first called to our attention by the results of hypophysectomy of the Spring peeper (*Pseudacris triseriata*) embryos. In these forms the melanophores were easily distinguished and revealed complete concentration of pigment at a time when the control animals were hatching from their jelly envelopes. Secondly preliminary experiments showed that head extracts of recently hatched *Rana pipiens* larvae yielded considerable amounts of melanophore-dispersing hormone when injected into hypophysectomized Anolis lizards, while the control body extracts gave a negative response. Histological examination of control pituitaries revealed no cytoplasmic differentiation.

These observations suggested, not only the presence of the melanophore-dispersing hormone in the undifferentiated gland, but also revealed its functional rôle during very early development.

This investigation was begun in 1939 with the view of using the chick embryo for test material, since in this species the cytological development of the pituitary had been described (1). In the meanwhile Kleinholz (2) confirmed the early appearance of this hormone in *Rana pipiens*, and Chen, Oldham and Geiling (3) reported its first detection from the 5-day-old Red-Rock chick embryo. While the latter determinations were of a qualitative nature, this paper presents the results of a quantitative assay of the melanophore-dispersing hormone throughout the entire development of the chick.

MATERIAL AND METHOD

The pituitaries from White Leghorns were used for test material. These glands were immersed in acetone immediately upon removal from the killed animal. In embryos younger than the 10th day of incubation the heads were placed in acetone and sufficiently hardened for dissection under the microscope. Later these dried glands were weighed. Since a pars intermedia is absent in the chicken (1) and other birds (4), and the pars buccalis can be easily removed without adhering tissues, the weight samples in all cases refer to the pars anterior alone. No pituitary weight determinations were attempted in embryos younger than the 12th day of incubation, since it was found impossible to separate these glands completely from surrounding membranes.

After complete dehydration the glands were stored in acetone. Extractions were made by heating the glands at 90°C. in a 0.1 N NaOH solution for 5 to 10 minutes. This solution was then neutralized with 0.1 N HCl using neutral red as indicator

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[1] Aided by the Wyoming Research Committee.

and used as soon as possible to guard against any possible deterioration. Subsequent dilutions were made with amphibian Ringer prior to each test. A total volume of 0.2 cc. of the dilution was injected intraperitoneally into each test animal, in order to assure as far as possible a uniform rate of absorption regardless of the concentration of hormone employed.

Two assays were performed on hypophysectomized lizards (*Anolis carolinensis*) according to the method previously described by Kleinholz and Rahn (5). An hypophysectomized animal is permanently green, since the pigment concentration of the dermal melanophores exposes the overlying chromatophores. Changes to a dark brown

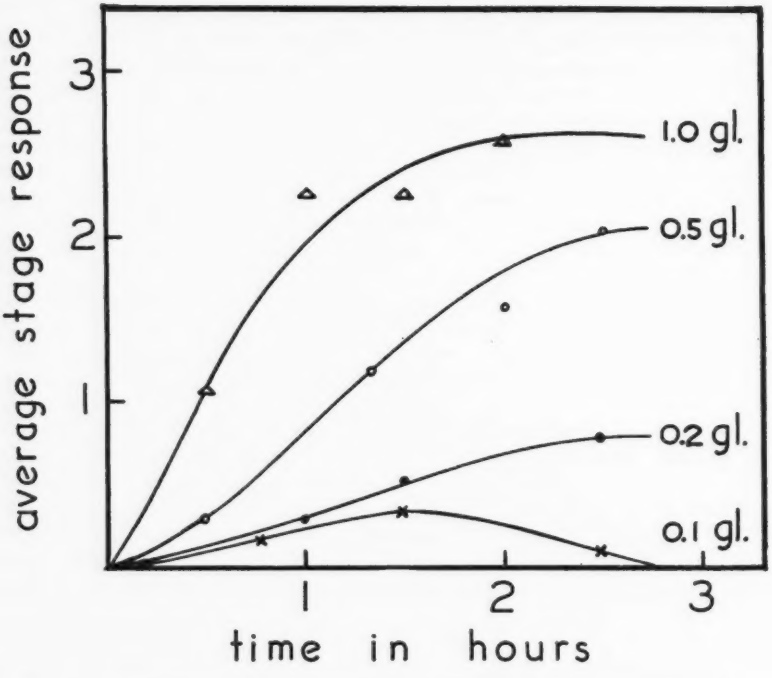

Fig. 1. Showing reaction time and average stage response of 10 hypophysectomized Anolis lizards receiving the equivalent of 1, 0.5, 0.2 and 0.1 pituitary of an embryo pituitary extract. The same 10 test animals were used in each run.

color occur only after administration of the melanophore-dispersing hormone. During this metachrosis the lizard passes through several distinct color changes which are readily recognized and are indicative of various hormone levels (fig. 1). In these assays the color scale was divided into 4 easily recognizable categories instead of 5 as previously described, since it was felt that a clear-cut distinction between *stages* 4 and 5 could not always be readily recognized. The color stages here employed are as follows (compare drawings in fig. 2):

Stage 0, completely pale green.
Stage 1, green with splotches of yellow on head, flank, and mid-dorsum.

Stage 2, yellowish brown (with some green).
Stage 3, light brown (no green remaining).
Stage 4, dark-brown to black.

Whenever the amount of pituitary material permitted, 10 lizards were injected intraperitoneally with a carefully measured dilution of pituitary extract, and color changes were recorded for each animal every half hour. At the end of 3 hours the animals had usually attained their maximal pigment dispersion. This maximal stage

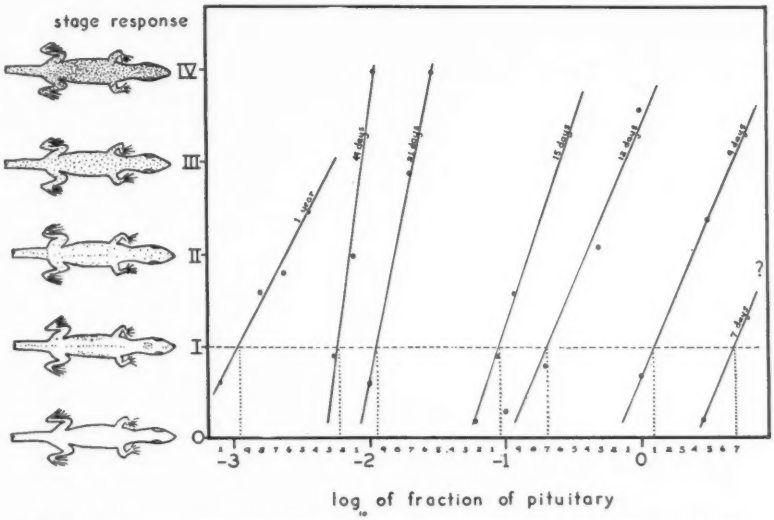

Fig. 2. Effect of various log concentrations of pituitaries of all age groups on the average stage response. The verticals dropped from the *stage 1* line intersect the abscissa at the pituitary concentration precisely equal to 1 Anolis Unit.

of each animal was classified as either 0, 1, 2, 3 or 4, intermediate shades being assigned to the nearest half integer. The 10 color responses were then totaled and averaged to give the 'average stage response' for the particular pituitary fraction injected. It may be seen from figure 1 that there exists a definite relationship between concentration of melanophore-dispersing hormone, onset of the response and the 'average stage response' obtained. The data in this figure were obtained from 4 runs using the same 10 animals in each assay.

If the average stage response for different dilutions of the same extract are plotted against the logarithms of these dilutions, nearly straight lines are obtained (fig. 2). This makes it possible to interpolate from the graph precisely the amount of pituitary gland necessary to bring about *stage 1*. Therefore, we can define from experimental determinations the Anolis Unit (A.U.) as the amount of hormone or pituitary tissue which injected into 10 hypophysectomized Anolis lizards will evoke an average stage response of 1.

In the earlier work on this problem many irregularities in color response were caused by the partial refractory state of melanophores of recently operated animals. After this fact was fully appreciated many of the earlier determinations had to be repeated in order to eliminate the possibility of this interference. Similar observations

on the refractory state have recently been recorded by Teague (6) who employed hypophysectomized frogs for his hormone assay. Consequently, a week or more was allowed to elapse after the operation before we considered an animal sufficiently sensitive for delicate tests. Hypophysectomized animals appeared to remain in excellent condition for at least a month when supplied with plenty of water or sugar water. They learned very readily to lap their daily supply of water from a pipette.

OBSERVATIONS

In order to study the hormone concentration of each age group increasing dilutions of the pituitary extract were made and assayed until responses were obtained

TABLE 1. ASSAY OF MELANOPHORE-DISPERSING HORMONE OF THE DEVELOPING CHICKEN PITUITARY

Age of chick	No. of glands extracted	Fraction and log of pituitary injected	No. of animals injected	Average stage response	Estimated fraction = 1 A.U.	A.U. per gland	Dry wt. single gland (γ)	A.U. per standard weight (100γ)
1 year	4	0.0004 (−3.40)	10	0.05	0.0011	909	1250	72.2
		0.0008 (−3.10)	10	0.6				
		0.0016 (−2.80)	10	1.5				
		0.0024 (−2.62)	10	1.8				
49 days	14	0.0058 (−2.24)	10	0.9	0.006	166	292	56.6
		0.0077 (−2.11)	8	2.0				
		0.0110 (−1.96)	3	4.0				
21 days	35	0.01 (−2.00)	5	0.6	0.011	90	150	60.0
		0.02 (−1.70)	7	2.9				
		0.03 (−1.52)	5	4.0				
		0.06 (−1.22)	5	3.8				
15 days	24	0.06 (−1.22)	10	0.2	0.09	11	86	12.7
		0.09 (−1.05)	10	0.9				
		0.12 (−0.92)	10	1.6				
12 days	28	0.1 (−1.00)	10	0.3	0.2	5	56	8.9
		0.2 (−0.70)	10	0.8				
		0.5 (−0.30)	10	2.1				
		1.0 (0.00)	10	3.6				
9 days	32	1.0 (0.00)	10	0.7	1.2	0.8		
		3.2 (0.51)	5	2.4				
7 days	30	3.0 (0.48)	10	0.2	5.0	0.2		
5 days	46	9.2	1	0.0				
		11.5	1	1.0				
		20.7	1	1.0				

averaging less than *stage 4*. In order to present in one graph (fig. 2) the various pituitary fractions of all age groups used (table 1) the logarithms of the amounts administered were plotted against the average stage response. As mentioned previously the Anolis Unit was then derived by dropping a vertical from the *stage 1* line and determining its intersection point on the abscissa. This gives a direct reading of the pituitary concentration equivalent of 1 Anolis Unit.

Table 1 summarizes all assays and calculations obtained. It should be noted that in the 7-day-embryo group, due to limited material, only one run was obtained. However, a line was plotted in order to obtain an estimation of the concentration equivalent to 1 Anolis Unit. It was assumed that this line would behave in a similar manner

to the straight lines obtained in other age groups (see fig. 2). The estimated units for the 7-day group consequently should be regarded with some caution.

In the 5-day-embryo group only 3 animals were injected with the hope of obtaining a qualitative response. As table 1 indicates, two of the animals receiving a total of more than 9 glands showed a positive *stage 1* response. No attempts were made to detect this hormone in younger animals.

DISCUSSION

This work presents a quantitative measurement of the melanophore-dispersing hormone of the pars anterior of the developing chicken pituitary. The earliest quali-

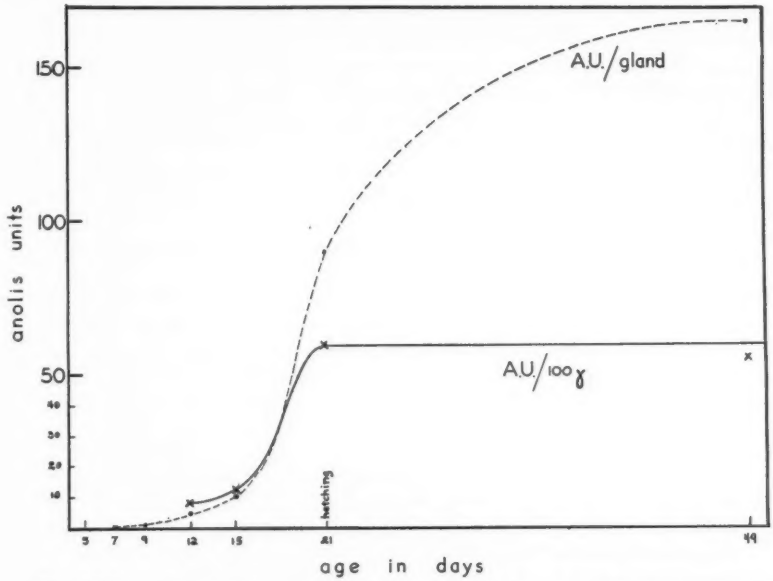

Fig. 3. MELANOPHORE-DISPERSING HORMONE CONCENTRATION PER GLAND AND PER STANDARD DRY WEIGHT (100γ) OF GLAND TISSUE DURING EMBRYONIC AND POST-NATAL DEVELOPMENT OF CHICKEN PITUITARY. There is no significant change in the concentration of hormone per unit weight after hatching, not even in the adult pituitary (compare table 1).

tative test was observed in the 5-day embryo and quantitative data were obtained commencing with the 7-day embryo. During the second half of the incubation period there is a very rapid increase in hormone concentration per unit volume. After hatching, however, the hormone concentration is not significantly changed, since the apparent increase in hormone content can be ascribed to the gain in weight of the gland. This is readily appreciated from a study of the last column in table 1 where the Anolis units per standard weight (100γ) are listed and from a study of figure 3.

Of great interest, of course, is the early manifestation of this hormone during ontogeny. It appears long before any cytological differentiation such as granulation or staining differences in cytoplasm can be detected. Rahn (1) has shown a slight basophilic differentiation beginning at 8 days of development and the first appearance of basophils and acidophils after 10 days of incubation. Chen, Oldham and Geiling (3) have likewise been impressed with the early hormone formation in the 5-day Red-

Rock (hybrid) chick embryo. They have shown further that just prior to the detection of the hormone the pars buccalis undergoes considerable growth and contacts for the first time the recent evagination of the infundibular process. From various evidences at hand (5, 7), it seems quite certain that in the chicken the pars anterior is the only site of the melanophore-dispersing hormone. The analysis of Chen, Oldham and Geiling as well as the experimental investigations of Blount (8) on the development of the pars intermedia in Amblystoma, indicate very strongly that the undifferentiated pars buccalis cells are capable of formation of the melanophore-dispersing hormone only after contact with the infundibular tissue has been made. It suggests a type of embryonic induction, with the infundibulum as inductor and the pars buccalis as reacting field.

Whether or not a similar situation exists in the frog or the mammal has not yet been ascertained, but it is clear from our own and Kleinholz's (2) observations for the frog and Snyder's (9) determination on the pig fetus that this hormone production likewise precedes histological differentiation of the pituitary gland.

The functional aspect of this hormone prior to pituitary differentiation has been demonstrated in urodele and anuran larvae. Blount's (10) observations on Amblystoma embryos and our observations on the peeper (*Pseudacris*) embryos revealed a pigment concentration in the melanophores at the time of hatching following previous removal of the pituitary primordium.

In the bird a function of this hormone has not yet been demonstrated, but its early appearance suggests possibly a metabolic rôle. However, another hormone of the pars anterior is known to exert a definite effect upon embryonic development. This is the thyrotropic factor which is necessary for the normal development of the thyroid (11) and appears as early as the 11th day of incubation. It is interesting to note that in this case the cytological differentiation of the pituitary (presence of acidophils and basophils) precedes by one day the first evidence of the hormone.

SUMMARY

The Anolis lizard test was employed to ascertain quantitatively the formation of the melanophore-dispersing hormone during the entire development of the chicken pituitary.

The earliest positive test was obtained from the White Leghorn embryo on the 5th day of incubation.

Quantitative tests were obtained from the 7th day on and show the greatest hormone increase per unit volume during the second half of the incubation period.

The maximum concentration was reached about hatching time. All further apparent increase could be ascribed to the size and weight increase of the pituitary gland.

The formation and significance of the early appearance of this hormone before the onset of cytological differentiation was discussed, and comparisons were made with similar observations for other animals.

REFERENCES

1. Rahn, J.: *J. Morph.* 64: 483. 1939.
2. Kleinholz, L. H.: *Biol. Bull.* 79: 432. 1940.
3. Chen, G., F. K. Oldham and E. M. K. Geiling: *Proc. Soc. Exper. Biol. & Med.* 45: 810. 1940.
4. Rahn, H., and B. T. Painter: *Anat. Rec.* 79: 297. 1941.
5. Kleinholz, L. H., and H. Rahn: *Anat. Rec.* 76: 157. 1940.
6. Teague, R. S.: *Endocrinology* 25: 962. 1939.
7. DeLawder, A. M., L. Tarr and E. M. K. Geiling: *J. Pharmacol. and Exper. Therap.* 51: 142. 1934.
8. Blount, R. F.: *Anat. Rec.* Suppl. 61: 6. 1935.
9. Snyder, F. F.: *Am. J. Anat.* 41: 399. 1928.
10. Blount, R. F.: *J. Exper. Zool.* 63: 113. 1932.
11. Martindale, F. M.: *Anat. Rec.* 79: 373. 1941.

EFFECT OF ADRENALECTOMY ON CARBOHYDRATE METABOLISM

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THE MARKED AMELIORATION OF PANCREATIC (1) and of phloridzin diabetes (2, 3) which is mediated by adrenalectomy has been explained at least in large part by the reduced capacity of adrenalectomized animals to convert protein to carbohydrate.

The experiments here presented investigate the extent to which carbohydrate metabolism itself is altered in the adrenalectomized animal.

EXPERIMENTAL

Influence of fasting. Twenty-four male rats, weighing from 150 to 200 gm. were adrenalectomized and maintained for the next 2½ days on their customary diet of

TABLE 1. EFFECT OF FASTING ON CARBOHYDRATE STORES OF INTACT AND ADRENALECTOMIZED RATS

6 animals per group

Duration of fast	Intact			Adrenalectomized		
	Blood glucose	Glycogen		Blood glucose	Glycogen	
		Liver	Rest of body		Liver	Rest of body
hr.	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*	*mg. %*
0	101	1830	381	77	2008	306
7	90	867	312	46	160	252
15	72	98	236	30	64	226
24	86	207	225	53	113	200

Purina dog chow but with 0.9% NaCl solution as drinking water. In groups of 6 they were killed when fully fed and after 7, 15 and 24 hours of fasting for examination of carbohydrate stores. Similar groups of intact rats were similarly killed and examined. The procedure of examination consisted of producing rapid anesthetization with nembutal and obtaining a blood sample from the femoral vein as soon as the limb stretch reflexes were lost. Immediately thereafter the liver was secured for freezing in a slush of carbon dioxide snow and ether, and then the whole remaining carcass was frozen in the same mixture. The analytical methods employed in this and the following experiments have been the same as in earlier work (2, 4, 5). The results of analyses are shown in table 1.

The adrenalectomized animals showed consistently lower blood sugar values. In both intact and adrenalectomized animals the lowest blood sugar values were obtained in the specimens taken at the 15th hour of fasting, a time when the lowest values for

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liver glycogen were obtained also. It is interesting that the ability to restore these low values for blood sugar and for liver glycogen are not entirely lost in the adrenalectomized animal; in the observations of glucose tolerance as recorded later in this paper, this restorative tendency is seen to be much more sluggish in the experimental than in the intact animal.

In agreement with previously published values, liver glycogen was found to be slightly higher in fed adrenalectomized animals than in controls; this finding deserves

TABLE 2a. EFFECT OF INTRAVENOUS GLUCOSE AND INSULIN ON CARBOHYDRATE IN RATS

Series 1. 6 rats per group

Group	Injected material	Glucose, mg. %						
		Blood			Gastrocnemius			Liver
		0 min.	90 min.	Increase	0 min.	90 min.	Increase	90 min.
Controls	Glucose	85	329	244	519	702	183	1268
	Glucose+ Insulin	88	181	93	485	809	324	105
Adrenal-ectomized	Glucose	57	346	289	406	527	121	275
	Glucose+ Insulin	59	182	123	409	660	251	110

TABLE 2b. Change in amt. of carbohydrate, expressed in mg. per 100 gm. of total body weight, calculated from data of table 2a

Group	Injected material	Increase in Glycogen			Increase in glucose	Carbohydrate	
		Liver	Rest of body	Total		Accounted for	Not accounted for
Controls	Glucose	52	92	144	122	266	234
	Glucose+ Insulin	−5	162	157	47	204	296
Adrenal-ectomized	Glucose	9	61	70	145	215	285
	Glucose+ Insulin	0	126	126	62	188	312

special note since, as will be seen below, fasting adrenalectomized animals do not store liver glycogen as readily as intact animals following intravenous injection of glucose. On fasting there is in the adrenalectomized animal a departure from the normal rate at which liver glycogen is lost. Since in the intact animal the total carbohydrate lost during 7 hours of fasting proves on calculation to be the amount necessary to supply the energy demands of the animal for about 1 hour, it can be said with reason that there exists in the intact fasting animal a mechanism for the conservation of carbohydrate stores. If this is the correct view to take of the facts then it would appear that the conserving mechanism is not fully operative in the adrenalectomized rat.

Intravenous glucose and insulin. Twenty-four-hour-fasted intact and adrenalectomized rats, the latter salt-maintained as before, and used 3 days postoperatively, were anesthetized, the kidneys ligated and a blood sample and one gastrocnemius obtained. They were then injected intravenously with 0.5 cc. of 50% glucose per 100

gm. of body weight; in half of these experiments this 0.5 cc. of glucose solution also contained 0.5 U. of insulin. This initial injection was repeated at 45 minutes; at 90 minutes another blood sample, the second gastrocnemius and the liver were secured for analysis. The results of these experiments, designated as *series* 1 are shown in table 2a, each value given being the average of 6 experiments.

A better idea of the changes involved is obtained if the actual amount of carbohydrate change following the injection is calculated. Table 2b shows such calculations. Certain assumptions are made in these calculations: *a*) that the total amount of glu-

TABLE 3. EFFECT OF INTRAVENOUS GLUCOSE AND INSULIN ON CARBOHYDRATE IN RATS

Series 2. Change in amts. of carbohydrate expressed as mg. per 100 gm. total body weight, calculated as explained in the text

Group	Injected material	No. of animals	Glucose retained	Increase in glycogen			Increase in glucose	Carbohydrate	
				Liver	Rest of body	Total		Accounted for	Not accounted for
Controls	Glucose	12	880	78±7[1]	114±11	192	121±7	313	567
	Glucose+ Insulin	11	946	4±3	181±14	185	82±8	267	679
Adrenalectomized	Glucose	4	904	52	46	98	204	302	602
	Glucose+ Insulin	4	936	1	114	115	162	277	659

[1] Standard error of difference.

cose in the body is obtained from the blood sugar by assuming this concentration to be distributed through half of the body weight; *b*) that the carcass glycogen exclusive of the liver is calculated from gastrocnemius glycogen by assuming this concentration of glycogen to be distributed through half of the body weight; by actual check this factor proved to be approximately 43% for fasted animals and 55% for fed animals, no great difference being noted between intact and adrenalectomized animals. It is conceded that the assumption may lead to false absolute values but it seems to afford a reasonable comparison of normal and adrenalectomized animals, and *c*) that the initial 24-hour-fasted liver glycogen is 200 mg.% for controls and 100 mg.% for adrenalectomized animals.

The most marked departure of the adrenalectomized animals from the controls is their failure to deposit glycogen under these conditions; this difference is so large as to leave the conclusion in no doubt. In both adrenalectomized and control animals liver glycogen was much less after glucose and insulin injection than when glucose alone was given. Although the adrenalectomized animals deposited less glycogen in gastrocnemii than did controls the insulin effect in this respect was about equal in the 2 sets of animals. The carbohydrate unaccounted for, which is shown in the right hand column of the table, represents in large measure the carbohydrate oxidized or converted; viewed in this way it would appear that the adrenalectomized animals oxidized or converted more of the injected glucose than did the controls.

A second series of experiments was made in which glucose or glucose and insulin was given by constant intravenous perfusion to lighly anesthetized 24-hour-fasted animals; the glucose was given as 25% solution at such a rate that for the 2-hour period each rat received per 100 gm. of body weight 1,000 mg. of glucose with or without 2 U. of insulin. In these experiments no initial samples were taken nor were the

kidneys ligated; the urine was collected and analyzed for glucose. At the end of the 2-hour period analyses were made of blood sugar. Using control values for the initial values of these substances, table 3 gives the calculations from the data which were obtained.

These data also indicate that the adrenalectomized animal provided with a carbohydrate plethora stores less and oxidizes or converts more carbohydrate than does the intact animal. In the experiments in which insulin also was given, the disappearance of carbohydrate was slightly less in the adrenalectomized group than in controls.

Intravenous glucose tolerance. The adrenalectomized rats used in these experiments were salt-maintained as previously and were used at the end of the third postoperative day; the experiments were begun 4 hours after removing food. In both series

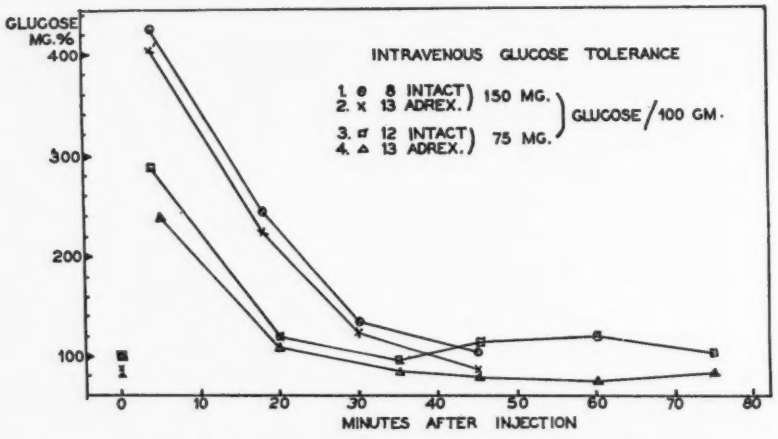

Fig. 1. Glucose tolerance in normal and adrenalectomized rats.

light nembutal anesthesia was used and the kidneys were ligated; in the first series 0.5 cc. of 30% glucose and in the second series 0.5 cc. of 15% glucose was given intravenously for each 100 gm. of body weight. Figure 1 shows the data which were obtained.

In both series it will be seen that as far as the return to original levels is concerned, the adrenalectomized animals have a normal glucose tolerance; the curves remain separated from one another by an amount approximately equal to the original difference in sugar levels. In *curves* 3 and 4 in which less sugar was given and in which the values were followed for a longer period of time, the rather marked rebound phenomenon seen in controls is not exhibited by the adrenalectomized animals; it is, however, not entirely absent. Since in the experiments above described, it appeared that the storage of carbohydrate was less in adrenalectomized animals than in the control, the curves here give support to the idea that in the presence of a carbohydrate plethora the oxidation of this foodstuff proceeds faster in adrenalectomized rats than in controls.

DISCUSSION

Because the subject of the adrenal cortex and carbohydrate metabolism has been covered by recent reviews (6, 7), extensive quotations need not here be given.

The rapid loss of carbohydrate by the adrenalectomized rat on fasting is in agreement with many reports in the literature and is the first line of evidence favoring more rapid oxidation of carbohydrate in these animals.

The relative failure of the adrenalectomized animal to deposit glycogen from injected glucose is definite and is in agreement with other reports (8–13). Deuel, Hallman, Murray and Samuels (14) feeding glucose to rats found normal absorption and storage. It should be pointed out that the fed adrenalectomized rats in table 1 had somewhat higher liver glycogen than did intact animals; the same finding has been previously reported (5, 11).

The increased disappearance of glucose in adrenalectomized animals could be explained on 4 grounds: as being due to *a*) decreased protein catabolism of adrenalectomized animals; *b*) to conversion to fat; *c*) an extra oxidation of carbohydrates; and *d*) an accumulation of carbohydrate intermediates. The last possibility as far as present knowledge is concerned is a hypothetical one and is not here considered; the other 3 are discussed in turn.

In earlier work on adrenalectomized rats it was shown that the mechanism for the formation of carbohydrate from protein was not fully operative. The difference in fasting nitrogen excretion per 100 gm. of rat per day was approximately 15 mg.; this would be equivalent to 100 mg. of protein capable of yielding 60 mg. of carbohydrate. Referring to the data of table 1 and calculated on this basis, the gluconeogenetic defect of adrenalectomized animals could account for 18 of the 38 mg. difference in carbohydrate disappearance between normal and adrenalectomized animals during the first 7 hours of fasting. It might possibly be of quantitative significance in simple fasting, but it could account for but 4 mg. of the 51 mg. difference in carbohydrate disappearance recorded for glucose-injected animals as shown in the calculations of table 2b; equally, gluconeogenesis could not affect the conclusions drawn from the glucose tolerance curves.

For manipulative reasons observations of respiratory metabolism could not be made in the injection experiments recorded above. Furthermore, the interpretation of such data obtained over short periods of time under abnormal and changing circumstances is open to objection and caution must be exercised in drawing too fine a conclusion from such limited respiratory data. Nevertheless, certain considerations recounted in the next paragraph indicate that for the possible exclusion of the factor of conversion of carbohydrate to fat as an explanation of the increased disappearance of injected carbohydrate in adrenalectomized animals, respiratory data would have value.

The carbohydrate unaccounted for in adrenalectomized animals (table 2b) exceeded that for controls by 51 mg. per 100 gm. Should this have been due to a conversion to fat, it can be calculated that there would be a rise in the R.Q. of approximately 0.1 regardless of even a considerable change in heat production; if the heat production did not increase, the oxygen usage would drop approximately 12%. On the other hand, if the extra 51 mg. were oxidized, thus sparing fat, the rise of R.Q. would be approximately 0.02. It may be seen, therefore, that the absence of a definite rise of R.Q. would exclude any appreciable conversion to fat; as regards oxygen usage, only a decrease would have relevant meaning and would indicate conversion to oxygen-poor substance.

Russell (11) has given respiratory data for normal and adrenalectomized rats both fasting and during glucose feeding. On the basis of the above discussion they do not give evidence for conversion to fat. Indeed, the R.Q. rise induced by feeding glucose is slightly less in adrenalectomized animals than in controls. By elimination, therefore, one arrives at increased oxidation as being the most reasonable explanation of the increased disappearance of carbohydrate in adrenalectomized animals.

When viewed against the decreased capacity for storage of injected glucose, the normal glucose tolerance curves here given constitute good evidence for enhanced carbohydrate oxidation after adrenalectomy. Because the amounts of glucose injected are

small and do not constitute a marked carbohydrate plethora, forced conversions are not reasonably to be considered, and increased oxidation would appear as the factor which balances off with decreased storage. Under various circumstances and with different animals the disposition of intravenously injected glucose by adrenalectomized animals has been found improved (15), unchanged (16), or impaired (12, 17, 18). It should be pointed out that in the present experiments salt-maintained animals which had only recently been operated were used, and that not only is the condition of the animal important but also the dose of glucose. This latter may be seen by reference to the data of table 2a or of table 3 where with larger doses, the circulating glucose is not removed as readily in adrenalectomized animals as it is in controls. It is obviously a matter of balance between storage and oxidation.

Fisher, Russell and Cori (19) concluded as a result of their work that hypophysectomized rats suffer a defect in the mechanism by which normally carbohydrate oxidation is depressed and carbohydrate levels thereby maintained during fasting. Russell (20) found for glucose-fed hypophysectomized rats that less glucose was stored and more was oxidized than in normal rats. The present findings as well as those of Russell (10, 11) lead to the same conclusions for the adrenalectomized rat. The results are consistent with those of Long, Katzin and Fry (6) who showed that cortical extract injected into glucose-fed normal rats brought about an increased glycogen deposition and decreased carbohydrate oxidation as compared to that resulting in glucose-fed normal rats not so injected.

SUMMARY

The adrenalectomized rat as compared to controls loses more carbohydrate in the early hours of fasting, stores less glycogen when intravenously injected with glucose or glucose and insulin, and has a normal intravenous glucose tolerance. A consideration of the data obtained in such experiments indicates that adrenalectomy results in an increased tendency to oxidize carbohydrate.

REFERENCES

1. Long, C. N. H., and F. D. W. Lukens: *J. Exper. Med.* 63: 465. 1936.
2. Evans, G.: *Am. J. Physiol.* 114: 297. 1936.
3. Wells, B. B., and E. C. Kendall: *Proc. Staff Meet., Mayo Clin.* 15: 565. 1940.
4. Evans, G.: *J. Physiol.* 82: 468. 1934.
5. Evans, G.: *Am. J. Physiol.* 110: 273. 1934.
6. Long, C. N. H., B. Katzin and E. G. Fry: *Endocrinology* 26: 309. 1940.
7. Soskin, S.: *Annual Rev. Physiol.* 3: 543. 1941.
8. Britton, S. W., and H. Silvette: *Am. J. Physiol.* 107: 190. 1934.
9. Britton, S. W., and E. L. Corey: *Am. J. Physiol.* 131: 790. 1941.
10. Russell, J. A.: *Proc. Soc. Exper. Biol. & Med.* 41: 626. 1939.
11. Russell, J. A.: *Am. J. Physiol.* 128: 552. 1940.
12. Fernandez, R., V. G. Foglia, L. F. Leloir and A. Novelli: *Compt. rend. Soc. de biol.* 115: 334. 1934.
13. Lewis, R. A., D. Kuhlman, C. Delbue, G. F. Koepf and G. W. Thorn: *Endocrinology* 27: 971. 1940.
14. Deuel, H. J., L. F. Hallman, S. Murray and L. T. Samuels: *J. Biol. Chem.* 119: 607. 1937.
15. Banerji, H., and C. Reid: *J. Physiol.* 81: 93. 1934.
16. Pollack, H., R. F. Mellet, J. L. Bollman and F. C. Mann: *Proc. Staff Meet., Mayo Clin.* 7: 722. 1932.
17. Samuels, L. T., H. F. Schott and H. A. Ball: *Am. J. Physiol.* 120: 649. 1937.
18. Hill, E., and A. E. Koehler: *Proc. Soc. Exper. Biol. & Med.* 30: 244. 1932.
19. Fisher, R. E., J. A. Russell and C. F. Cori: *J. Biol. Chem.* 115: 627. 1936.
20. Russell, J. A.: *Am. J. Physiol.* 121: 755. 1938.

DEAMINATION OF DL-ALANINE IN THE ADRENALECTOMIZED RAT

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THE ADRENALECTOMIZED ANIMAL has a limited ability to convert protein to carbohydrate (1, 2, 3). It is, therefore, of interest to examine the capacity of these animals to de-aminate injected amino acid.

EXPERIMENTAL

Alanine injection. All animals were 24-hour fasted male rats, weighing between 150–200 gm. The adrenalectomized animals were maintained on 1% NaCl as drinking water and were used on the third day postoperatively. After anesthetization with nembutal, the abdomen was opened and the kidneys ligated. Initial blood samples were taken and the animals then slowly injected intravenously with 75 mg. of two-thirds neutralized dl-alanine per 100 gm. of body weight. Blood samples were drawn at 30 and 90 minutes after injection. Blood urea nitrogen and amino acid nitrogen were determined on tungstic acid filtrates made from whole blood. The method used for amino acid nitrogen was based on the colorimetric method of Folin. The Evelyn photoelectric colorimeter was employed. The results are given in table 1. The variations shown are the standard errors of the differences.

TABLE 1. DEAMINATION OF INTRAVENOUS ALANINE IN NORMAL AND ADRENALECTOMIZED RATS

(a) Series I.	No. of Animals	Blood Amino Acid N, mg. %				
		0 min.	30 min.	90 min.	30–90 min.	90–0 min.
Controls	12	10.61	24.34	17.55	6.79±0.58	6.94±0.70
Adrenalectomized	12	10.44	24.70	17.98	6.72±0.55	7.54±1.02

(b) Series II.	No. of Animals	Blood Amino N, mg. % 90 min.	Blood Urea N, mg. %		
			0 min.	90 min.	90–0 min.
Controls	12	17.23	13.13	19.92	6.79±0.49
Adrenalectomized	14	17.62	13.96	20.46	6.50±0.5

The amount of alanine injected was shown to be well within the limits tolerated by adrenalectomized animals which were found to be more susceptible to the toxic effects than were normal animals. The tying of the kidneys was decided upon because of analytical difficulties with urine that would have rendered the results uncertain. It was determined, however, that the deamination of injected alanine occurring in animals with tied kidneys was at least 90% of that which occurred in intact animals; for this reason it would appear that the preparations decided upon were suitable. A preliminary series of experiments using 6 normal and 6 adrenalectomized animals in which

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frequent blood samples were drawn showed that for the first 30 minutes after injection the blood amino acid nitrogen values fell rapidly on a curved plot as if still being distributed throughout the body. The values obtained from 30 to 90 minutes fell at a uniform rate with the passage of time; for this reason, the 0, 30, and 90 minute values were chosen for analysis.

Observations defining the condition of the animals used. The present paper concludes the report of earlier work on the adrenalectomized rat. It is appropriate, therefore, to record observations which have been made on the condition of the salt-maintained adrenalectomized rat on the third day postoperatively in which abnormality of gluconeogenesis from protein (1) and abnormal disposition of injected carbohydrate (4) have been demonstrated. Because such different functions as control of electrolytes and regulation of protein-carbohydrate metabolism appear to be resident in one endocrine organ, it is important that workers in one field should as far as possible define the condition of the adrenalectomized animal in terms of the criteria of the workers in other fields. The manifestations of adrenal insufficiency as they have been thought of in connection with electrolyte and water disturbances include the following: *a*) muscular weakness and loss of activity, *b*) refusal of food, *c*) loss of body weight, *d*) lowered body temperature, *e*) lowered oxygen consumption, *f*) increased blood urea nitrogen, g) hemoconcentration, *h*) decreased serum sodium, *i*) increased serum potassium. The first 6 of these criteria have been previously (1) commented upon for the animals in question. They are active, healthy in appearance, eat well and maintain weight, have rectal temperatures of 37–37.5° C., show an oxygen usage 4% below that of intact animals, and have blood nonprotein nitrogen values that do not exceed by 5 mg.% the values for controls. In the data of table 1b it can be seen that the animals have essentially normal blood urea nitrogen values. As regards hemoconcentration, intravenous injection of brilliant vital red gave an apparent blood volume of 7.02% of body weight in 6 normals and 6.90% in 6 adrenalectomized rats salt-maintained for 4 days. Both groups before injection were anesthetized, the kidneys tied, and the gastrointestinal tract and spleen removed, care being taken to leave intact the arterial supply to the liver. It is probably to be conceded that this procedure in itself constitutes a test of the adrenalectomized animal. In like groups of the same preparations, the extracellular fluid volume as determined by sucrose injection was 24.4% of body weight for adrenalectomized and 25.5% for controls; injected urea distributed itself throughout 73.9% of body weight in adrenalectomized and 72.2% in controls; dry weight/wet weight ratios for lungs were 0.210 and 0.223 and for gastrocnemii were 0.238 and 0.243 for adrenalectomized and for controls, respectively, in each case. The differences are small and probably not significant but indicate, that the adrenalectomized preparations had a slightly smaller blood and extracellular fluid volume with some intracellular hydration. When 1 cc. of blood per 100 gm. of initial body weight was withdrawn from such preparations and replaced by 1 cc. of 4% NaCl, the sodium value of the serum 45 minutes later was 6.2 m. eq. per liter higher in adrenalectomized and 6.0 m. eq. per liter higher in controls than before the salt injection. The ratio of hemoglobin before blood withdrawal and salt injection, to hemoglobin 45 minutes after withdrawal and injection was 1.148 for adrenalectomized and 1.159 for controls. The departure from normal is seen to be small.

Sodium determinations were done in triplicate on pooled blood from 10 animals and gave values of 135.5 m. eq. per liter for adrenalectomized and 137.4 m. eq. per liter for controls. Potassium values of 5.6 m. eq. per liter were obtained for adrenalectomized animals as compared to 5.1 m. eq. per liter for controls.

The observations give a surprisingly normal picture for these adrenalectomized preparations; with this must be placed the normal deamination here recorded.

On the other hand these adrenalectomized animals have shown marked inadequa-

cies. When blood to the amount of approximately 2 cc. per 100 gm. of body weight was withdrawn, they frequently suffered sudden vascular collapse and the animals often died. The blood flow through the leg was very small. Also when glucose had been fed by mouth, it had been found that blood was obtained with difficulty from the femoral vein; on opening the abdomen of such animals, the gastrointestinal tract was found to be filled with fluid. At this time intact rats had absorbed the fed glucose and had empty gastrointestinal tracts. The toxic effect of injected alanine, leading to the death of the animals, was found to occur at a much lower level for adrenalectomized than for intact animals.

DISCUSSION

The results indicate that in the adrenalectomized and functionally nephrectomized rat, failure of normal deamination does not enter into the diminished ability of the animal to convert protein to carbohydrate. This is in agreement with the results of Wells and Kendall (3) who showed in phloridzinized rats that the sugar production from casein proceeded as well in the adrenalectomized as in the intact animals. Samuels, Butts, Schott, and Ball (5) found in adrenalectomized, as compared to intact animals, a smaller rise of liver glycogen following alanine feeding; they were, however, cautious in drawing conclusions regarding the rate of deamination. Such a criterion of alanine conversion was not considered for the present experiments because of the demonstration (4) that adrenalectomized rats do not store liver glycogen normally from injected glucose.

Russell and Wilhelmi (6) using kidney tissue in the Warburg apparatus and either dl-alanine or l(+)-glutamic acid as substrate, found a smaller ammonia production in tissue from adrenalectomized as compared to that from intact rats. Treatment of the adrenalectomized rats with either adrenal cortical extract or desoxycorticosterone brought the ammonia production of the kidney tissue up to that of controls. Jiminiz-Diaz (7) had also found a lowered deamination rate for kidney tissue from adrenalectomized animals. The findings of Jiminez-Diaz and of Russell and Wilhelmi cannot be thought of as discrepant with the conclusions of this paper because the present experiments exclude the participation of the kidney which in any case was shown to have but a minor rôle in the total deamination of injected alanine.

The demonstration of a relatively normal electrolyte status for the adrenalectomized animals used in this and other work does not prove either that the protein-carbohydrate disturbance precedes the electrolyte changes or the reverse. It does, however, indicate that protein-carbohydrate abnormalities (1, 4) occur in animals which are not suffering from marked adrenal insufficiency.

SUMMARY

In adrenalectomized, functionally nephrectomized rats, the rate of disappearance of intravenously injected dl-alanine and the extent of the consequent urea formation are the same as found for non-adrenalectomized controls.

The condition of the salt-maintained adrenalectomized animals used in this and previous work is further defined.

REFERENCES

1. Evans, G.: *Am. J. Physiol.* 114: 297. 1936.
2. Long, C. N. H., and F. D. W. Lukens: *J. Exper. Med.* 63: 465. 1936.
3. Wells, B. B., and E. C. Kendall: *Proc. Staff Meet. Mayo Clin.* 15: 565. 1940.
4. Evans, G.: *Endocrinology* 29: 731. 1941.
5. Samuels, L. T., J. S. Butts, H. F. Schott and H. A. Ball: *Proc. Soc. Exper. Biol. & Med.* 35: 538. 1937.
6. Russell, J. A., and A. E. Wilhelmi: *J. Biol. Chem.* 137: 713. 1941.
7. Jiminez-Diaz, C.: *Lancet* 2: 1135. 1936.

EFFICACY OF DESOXYCORTICOSTERONE ACETATE AS REPLACEMENT THERAPY IN ADRENALECTOMIZED DOGS

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THE SYNTHETIC STEROID DESOXYCORTICOSTERONE ACETATE (D.C.A.) is now used extensively as partial or complete replacement therapy in adrenalectomized animals and in patients with Addison's disease. Considerable evidence suggests, however, that its physiological action is not identical in all respects with that of the natural cortical extract. For instance, its action on the kidney of both the intact and adrenalectomized animal in conserving Na and Cl and releasing K is even more powerful than that of cortical extract (1–5). By virtue of this renal action, the serum Na concentration has, in some cases, been elevated above normal (3–6). Also, the serum K concentration has, by large daily doses given over a considerable time interval, been appreciably lowered, to the point at which toxic symptoms appear (3–5). Neither of these blood electrolyte changes have been observed in animals given cortical extract therapy. D.C.A. induces a large positive water balance and a rapid gain in blood volume and body weight (2, 4, 7, 8). It also has a striking action in elevating the blood pressure, which may even attain hypertensive levels (2, 4, 7, 8).

With regard to certain other established functions of the adrenal cortex, however, such as the maintenance of the capacity for normal muscular work (9), the antagonism to insulin (12) and a basic rôle in gluconeogensis (10–12), D.C.A. exhibits no positive action. Despite its efficacy in protecting the adrenalectomized animal against circulatory collapse following hemorrhage, muscle trauma and epinephrine infusions, it will not prevent circulatory failure after all types of shock-producing procedures (13), in this respect being unlike cortical extract.

Some of the unusual symptoms which follow the use of D.C.A. are perhaps attributable to overdosage, others to an improper dietary regime, and others to the inherent properties of the hormone itself. The relative importance of these three factors is not well defined. During the past year we have maintained a colony of adrenalectomized dogs routinely on various dosage levels of D.C.A. From our experience with its use have arisen several more or less isolated points which, while they do not constitute a complete study, still seem to have direct bearing on the question of efficacy of D.C.A. as partial or complete replacement therapy in the adrenalectomized dog.

METHODS

The studies to be reported comprise observations on 25 adrenalectomized dogs (8–12 kg. body weight) maintained for various lengths of time on D.C.A.[1] in oil given intramuscularly. These dogs were fed either Ken-L-Ration (400–600 gm. per day) or cut meat scraps (300–500 gm. per day), with cod liver oil and yeast added, and a

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[1] We are indebted to the Ciba Pharmaceutical Products, Inc., Summit, N. J., for generous supplies of the desoxycorticosterone acetate (Percorten) used in these experiments.

supplement of 2 gm. NaCl usually included. Under these conditions, the usual maintenance requirement of D.C.A. was from 0.25 to 0.5 mg. per dog per day. The major portion of the data were obtained on animals given from 0.25 to 2 mg. per day. To study the effect of larger D.C.A. doses, a detailed metabolic study on two dogs was made, each receiving 1 mg. per kg. body weight per day, or about 40 times the maintenance requirement. Larger dosage levels than this have not been employed.

Blood pressures were followed routinely (14). At selected time intervals, complete blood and urine analyses were made, using the following technics (15): Cl, modified Volhard-Harvey, Van Slyke and Sendroy; Na, Butler and Tuthill; K, Kramer and Tisdall; hematocrit, capillary tube; hemoglobin, Newcomer; urea nitrogen, Van Slyke and Cullen; blood sugar, Benedict; serum proteins, Bierry and Vivarvo.

RESULTS

Electrolyte balance. The highly efficient control of D.C.A. over the renal excretion of electrolytes is illustrated by the data obtained from a representative experiment given in figure 1. Although complete blood and urine analyses were made, only the more pertinent parts of the data have been included. In this experiment, the NaCl intake was varied by the addition of salt supplements and the use of a synthetic salt-free diet.[2] Rapid readjustment in electrolyte excretion followed each change in intake, so that there was never more than a temporary imbalance between intake and output. For example, on the salt-free regime, a small loss of Na and Cl on the first day was followed by the excretion of an almost salt-free urine. The electrolyte deficit incurred over this period was almost entirely restored on the first day of NaCl resumption. Hence regardless of intake, the renal action of D.C.A. effectively guarded against any serious deficit of Na and Cl.

We have not observed serum Na and Cl concentrations definitely above normal in dogs receiving either minimal maintenance or larger amounts of D.C.A. with the standard dietary regime. With larger D.C.A. dosage and higher salt intake (fig. 1), the serum Na concentrations did show a small and probably significant rise above normal. This elevated concentration was lost on the salt-free regime, and regained on the resumption of a high salt intake.

The serum K concentrations of D.C.A. maintained dogs tended to be almost consistently in the lower range of normal. In fact, an animal in which D.C.A. had been substituted for cortical extract therapy usually showed some decline in serum K level. With large D.C.A. dosage, this decline was often fairly rapid (fig. 1). Yet neither definitely subnormal concentrations nor abnormal symptoms, such as those found by Kuhlman et al. (5) with much larger doses of D.C.A., which might be attributed to low serum K concentrations, have been observed in these dogs.

Blood sugar. Although D.C.A. reputedly has no direct rôle in carbohydrate metabolism, the absorption and storage of ingested sugar is normal in the adrenalectomized animal on maintenance D.C.A. therapy (18, 19). The blood sugar level of our animals on minimal maintenance amounts of D.C.A. has not been abnormal. With larger doses of D.C.A. (1–9 mg. per day), however, the blood sugar level was often in the lower range of normal or distinctly sub-normal. This change in sugar level was usually not reflected by any detectable change in the physical signs of general health and vigor of the animal. The low blood sugar was usually responsive to the injection of glucose, or better, the addition of a glucose supplement to the diet (fig. 1). The extra sugar intake did not always permanently correct the deficiency, however. We have had 4 dogs which showed a quite unexpected spontaneous development of hypoglycemic convulsions. It should perhaps be stressed that in all of these cases,

[2] A modified (16) Cogwill's casein diet no. III (17), with a salt mixture free from Na, Cl and K.

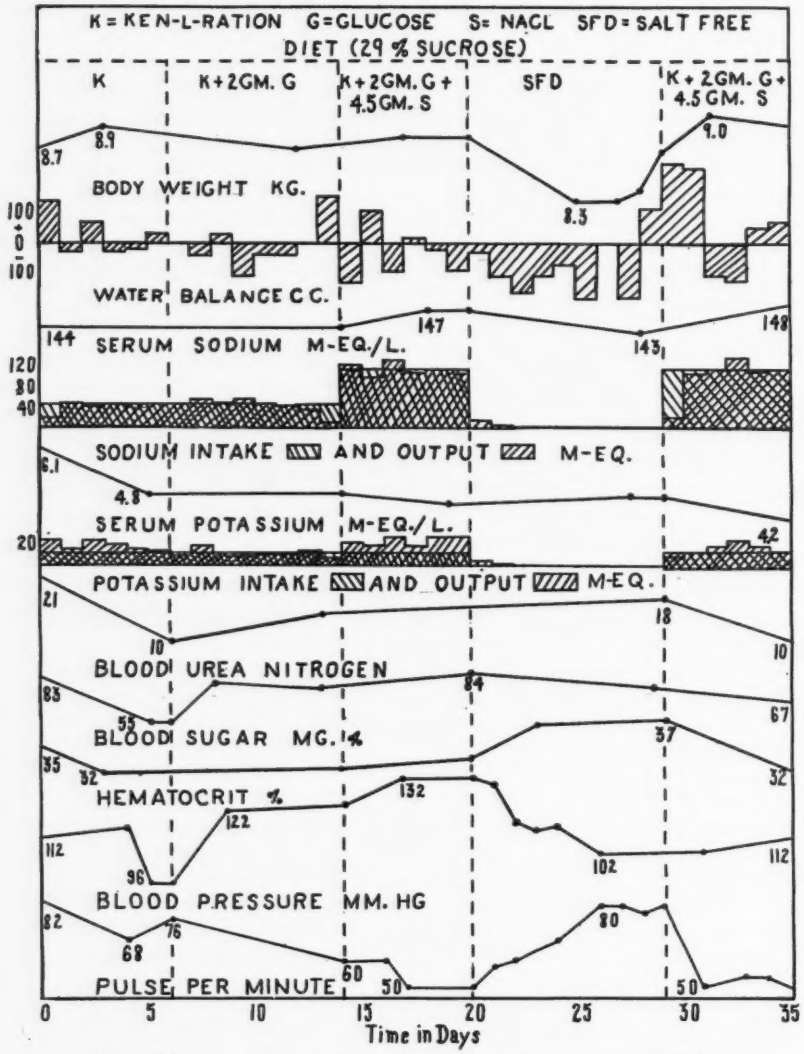

Fig. 1. METABOLIC DATA ON ADRENALECTOMIZED DOG RECEIVING 1 MG. OF D.C.A. PER KG. OF BODY WEIGHT PER DAY.

the dogs were receiving 2 mg. or more of D.C.A. per day. The administration of glucose relieved the hypoglycemia.

Blood urea nitrogen. As to be expected, D.C.A. therapy will rapidly reduce the elevated blood non-protein nitrogen level of the patient with Addison's disease or of the adrenalectomized animal receiving inadequate hormone treatment, to normal (1, 4–8). Further, a small decline from normal levels has been reported in intact dogs given large doses of D.C.A. (5). The life span of rats following complete nephrectomy can be prolonged by D.C.A. treatment (20), the rise in blood N.P.N. being less pronounced than in the control animals.

One of the most striking changes observed in the blood picture of our adrenalectomized dogs on D.C.A. therapy has been a decline in blood urea nitrogen level, an effect which has also been produced in intact dogs. The reduction may be as large as 60% (table 1). It was usually slow and progressive, reaching the lowest value in 10 to 30 days. In some cases the changes tended to be more rapid, but not necessarily of greater magnitude, with higher dosage levels of D.C.A. For instance, a significant lowering of the blood urea nitrogen level has been observed within 24 hours after the injection of 10 to 20 mg. of D.C.A. An animal showing a full decline in urea may,

TABLE 1. BLOOD UREA NITROGEN (MG. PER 100 CC.). CHANGES IN ADRENALECTOMIZED AND INTACT DOGS RECEIVING D.C.A.

	Adrenalectomized Dogs										
Dog No.	1	2	3	4	5	6	7	8	9	10	11
Dose, mg./day	.5	.5	.5	.5	1	1	1	2	2	2	5
Initial	26.0	22.3	25.0	17.0	29.4	22.3	22.1	23.5	22.0	22.1	24.2
Final	22.3	6.8	12.5	9.0	16.7	14.2	16.2	9.3	20.8	12.3	18.4
% change	14	69	50	47	43	35	26	60	9	44	24

	Adrenalectomized Dogs					Intact Dogs					
Dog No.	12	13	14	15	Av.	1	2	3	4	5	Av.
Dose, mg./day	6	9	10	10		2	2	2	4	5	
Initial	20.5	21.1	14.2	16.7	21.9	18.4	13.6	16.7	19.0	22.4	18.1
Final	9.7	10.0	9.3	10.0	13.3	7.4	6.2	12.1	11.2	12.6	9.7
% change	53	52	35	40	41	59	55	29	41	43	46

on continuation of D.C.A. therapy, show a spontaneous rise to the previous level, but this is the exception. Upon the replacement of D.C.A. therapy with cortical extract, the blood urea nitrogen levels tend to return to normal.

The factors underlying this fall in blood urea are not clear. In some cases, especially where the decline is rapid, it could perhaps be explained by the accompanying dilution of extracellular fluids. Usually the urea change was too large to be entirely accredited to a dilution effect. Further, an occasional dog has shown a large urea change with no observable blood dilution, and conversely, a normal urea level with extensive blood dilution.

At times it would seem that changes in the level of urea nitrogen were related to variations in the blood sugar level (fig. 1), but this is not always apparent. The decline in urea might, of course, have been reflecting a depression in protein catabolism of the body, with a resultant impaired conversion of protein to carbohydrate (10, 12).

Body weight, blood dilution. Often the first change manifest in the adrenalectomized dog placed on D.C.A. therapy was a gain in body weight, associated with a fall in blood hematocrit and hemoglobin values, and in serum protein levels. In fact, the weight changes show fairly good agreement with the body water gain as calculated from these indices of blood dilution (fig. 1, table 2). This weight gain was of the same order of magnitude with salt intakes ranging from 1 gm. to 6 gm. per day and D.C.A. dosages from 0.5 to 9 mg. per day. The weight gain and dilution changes were progressive for the first 5 to 10 days, after which a stable level was attained and held as long as the D.C.A. therapy was continued. No evidence of edema or related conditions which might be ascribed to excessive gains in body water were found in these animals.

Blood pressure, pulse rate. The patient with Addison's disease placed on D.C.A. therapy shows a rise in blood pressure which may, in some cases, ascend above

TABLE 2. BLOOD DILUTION CHANGES IN ADRENALECTOMIZED DOGS PLACED ON D.C.A. THERAPY

Dog No.	Therapy	Dose	Hemotocrit	Hemoglobin	Serum Protein	Body Wt.	Theoretical[1] Body Weight Gain
		mg./day	%	gm. %	%	kg.	kg.
1	C.E.[2]		37.6	11.5	6.6	10.6	
	D.C.A.	.5	34.4	11.0	6.0	10.8	0.3
2	C.E.		34.5	11.5	7.0	9.1	
	D.C.A.	.5	33.6	10.6	6.9	9.4	0.3
3	C.E.		44.3	14.0	7.5	9.5	
	D.C.A.	.5	38.2	11.5	6.5	10.1	1.1
4	C.E.		32.5	9.6	5.5	9.1	
	D.C.A.	1	29.6	9.4	5.2	9.7	0.5
5	C.E.		43.8	15.0	7.0	10.0	
	D.C.A.	1	35.1	12.0	6.0	10.8	1.4
6	C.E.		29.7	8.3	6.9	12.9	
	D.C.A.	1	26.7	7.8	6.3	13.7	0.9
7	C.E.		38.7	12.4	6.2	10.9	
	D.C.A.	1	36.7	10.7	6.0	10.8	0.6
8	C.E.		40.2	13.0	6.9	10.0	
	D.C.A.	2	36.4	11.0	6.0	10.5	1.1
9	C.E.		39.9	10.8	6.6	12.8	
	D.C.A.	6	37.6	10.3	6.2	13.3	0.4
10	C.E.		37.0	10.6		8.5	
	D.C.A.	9	31.9	9.8		8.9	0.7
Av.			37.8	12.7	6.7	10.3	
			34.1	11.5	6.0	10.8	0.7

[1] Based on calculations from hematocrit, hemoglobin and serum protein changes.
[2] On maintenance levels of cortical extract.

normal to hypertensive levels (2, 4, 7, 8). Moderate elevations in pressure have been produced in intact dogs with large doses of D.C.A. (5). Pressure rises have been consistently observed in our adrenalectomized dogs on D.C.A. therapy (table 3). With the standard diet, both the rapidity and the extent of the rise seemed independent of the D.C.A. dosage employed. From the data presented in figure 1 it might seem that the pressure rise was to be correlated with the salt intake. It is certainly true that the elevated pressure may be reduced by the use of a diet free from salt. With salt intakes of 2 to 6 gm. per day, however, variations in intake have not obviously influenced the rapidity or extent of the pressure rise. An interval of 6 to 20 days is usually required to produce the maximum pressure elevation. The blood pressure of the dog

TABLE 3. BLOOD PRESSURE AND PULSE RATE CHANGES IN ADRENALECTOMIZED DOGS PLACED ON D.C.A. THERAPY

Dog No.	Cortical Extract		D.C.A.				Change in Pressure	Change in Pulse
	Blood Pressure	Pulse	Time on D.C.A.	Dose	Blood Pressure	Pulse		
	mm. Hg	per/min	days	mg./day	mm. Hg	/min.	mm. Hg	/min.
1	110	80	20	.5	160	60	50	−20
2	102	76	8	.5	126	68	24	− 8
3	105	80	10	1	128	64	23	−16
4	105	88	7	1	130	64	25	−24
5	100	84	6	1	120	80	20	− 4
6	112	92	10	2	147	64	35	−28
7	114	82	7	6	131	50	17	−32
8	112	64	8	9	132	44	20	−20
Av.	108	81	10		134	62	26	−19

cited in figure 1 reached a peak in 30 days, an interval perhaps extended by the remission associated with a fall in blood sugar in the early period. The pressure was lowered to below normal on the salt-free regime. When salt was restored, the pressure again rose. Despite the high salt intake, the time interval was not sufficient to produce a rise to the previous peak pressure.

When cortical extract was substituted for D.C.A. therapy, the blood pressure declined. Untoward symptoms which might be ascribed to the elevated pressure with D.C.A. have not been observed.

Associated with the pressure rise was an almost invariable fall in pulse rate to sub-normal levels. Although the two seemed related, they did not always change at the same time nor in proportional amounts. In fact, changes in pulse rate showed more positive correlation with changes in body weight, hematocrit and hemoglobin values, as though the pulse were reflecting changes in blood volume. In some cases, the pulse rate was not immediately restored to normal on the resumption of cortical extract therapy, even though the blood pressure was reduced.

SUMMARY AND CONCLUSIONS

For the most part, D.C.A. therapy has proved a fairly reliable replacement therapy in adrenalectomized dogs. It maintained normal serum electrolyte concentrations and normal electrolyte excretion under various experimental conditions. The serum Na concentration has, with high salt intake, even been raised above normal. Serum K concentrations, while low, were in general within the normal range. The blood urea nitrogen level was usually lowered, a change possibly associated with a depression of protein catabolism. The body weight was elevated, with the gain fairly well correlated with the dilution of body fluids as indicated by lowered hematocrit, hemoglobin, and serum protein values. The only definitely untoward effects of D.C.A. observed have been the rise in blood pressure, which was not extreme, and the tendency toward a low blood sugar level, with the occassional sudden development of hypoglycemia. When minimal maintenance levels of D.C.A. are used, this danger of hypoglycemia is minimized.

REFERENCES

1. THORN, G. W., AND H. EISENBERG: *Endocrinology* 25: 39. 1939.
2. THORN, G. W., R. P. HOWARD AND K. EMERSON: *J. Clin. Investigation* 18: 449. 1939.
3. WELLS, B. B., AND E. C. KENDALL: *Proc. Staff Meet. Mayo Clin.* 15: 133. 1940.
4. LOEB, R. F., D. W. ATCHLEY, J. W. FERREBEE AND C. RAGAN: *Trans. Assoc. Am. Physicians* 54: 285, 1939
5. KUHLMAN, D., C. RAGAN, J. W. FERREBEE, D. W. ATCHLEY AND R. F. LOEB: *Science* 90: 496. 1939.
6. HARTMAN, F. A., L. A. LEWIS, J. E. GABRIEL, H. J. SPOOR AND K. A. BROWNELL: *Endocrinology* 27: 287. 1940.
7. RYAN, E. J., AND E. P. MCCULLAGH: *Cleveland Clin. Quart.* 7: 19. 1940.
8. WILDER, R. M.: *Proc. Staff Meet. Mayo Clin.* 15: 273. 1940.
9. INGLE, D. J.: *Endocrinology* 26: 472. 1940.
10. LONG, C. N. H., B. KATZIN AND E. G. FRY: *Endocrinology* 26: 309. 1940.
11. WELLS, B. B., AND A. CHAPMAN: *Proc. Staff Meet. Mayo Clin.* 15: 503. 1940.
12. THORN, G. W., G. F. KOEPF, R. A. LEWIS AND E. F. OLSEN: *J. Clin. Investigation* 19: 813. 1940.
13. SWINGLE, W. W., H. W. HAYS, J. W. REMINGTON, W. D. COLLINGS AND W. M. PARKINS: *Am. J. Physiol.* 132: 249. 1941.
14. PARKINS, W. M.: *Am. J. Physiol.* 107: 518. 1934.
15. PETERS, J. P., AND D. D. Van Slyke: Quantitative Clinical Chemistry, Vol. II. Williams and Wilkins. Baltimore.
16. DRILL, V. A.: *Am. J. Physiol.* 132: 629. 1941.
17. COWGILL, G. R.: *Am. J. Physiol.* 85: 45. 1928.
18. ANDERSON, E., AND V. HERRING: *Proc. Soc. Exper. Biol. & Med.* 43: 363. 1940.
19. BRITTON, S. W., AND E. L. COREY: *Am. J. Physiol.* 129: P316. 1940.
20. SELYE, H., AND K. NIELSEN: *Proc. Soc. Exper. Biol. & Med.* 46: 541. 1941.

EFFECTS OF ADRENALECTOMY AND DESOXYCORTICOSTERONE SUBSTITUTION THERAPY ON THE CASTRATED RAT PROSTATE

EVIDENCE FOR AN ANDROMIMETIC FUNCTION OF THE IMMATURE RAT ADRENAL

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THE ISOLATION OF AN ANDROGENIC STEROL, adrenosterone, from adrenal cortex extracts (1) provided a definite basis for relating the activities of the normal gland to the masculinizing effects of certain cortical tumors. The present paper has as its object an evaluation of the degree to which the normal cortex functions as a source of physiologically active androgens by means of a study of the effect of adrenalectomy on the prostates of castrated rats.[1] In analyzing the effects of adrenalectomy on the male reproductive system it has been considered of importance to maintain the general health of the animals as nearly normal as possible by the administration of desoxycorticosterone acetate.[2]

It has previously been shown that there occurs an anomalous maintenance of well differentiated epithelium in the prostate and seminal vesicles of rats and mice which were castrated at 3 weeks of age (2, 3, 4). In adults the maintenance of such conditions is dependent on the presence of androgens (5, 6). In young rodents these conditions are associated with histological differentiations in the adrenal cortex and hence it was suggested by the author (3, 4, 7) that these reactions in male secondary sexual tissues might be indicators of an adrenal andromimetic activity, which appears to be particularly well developed during this period of life. The effect of adrenalectomy on the maintenance of the immature castrated rat prostate will now be reported.

It has been shown in a number of laboratories that degenerative changes may occur in a variety of tissues in adrenal insufficiency. Hence observations of degenerative phenomena in the reproductive tract after adrenalectomy must be interpreted with caution. Thus, fatty degeneration in kidney tubules has been frequently noted (8–13) as well as ulceration of gastric mucosa, and necrosis of liver cords (9, 10). However, the degenerative changes in the kidney are not necessarily associated with death from adrenal insufficiency (14). In this connection the observations of Marshall and Davis (15) are particularly illuminating. These investigators found that subjecting the kidneys of adrenalectomized cats to functional stress by injections of urea and creatinine caused visible degenerative changes to become manifest in the tubule cells. Normal control cats showed the same change to a much less degree.

Some observations have been made on the effects of adrenalectomy on the intact male reproductive system. Reduction in the size of seminal vesicles and prostate with histological

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[1] A preliminary report of this work was presented in *Amer. J. Physiol.* 133: P336. 1941.

[2] The crystalline desoxycorticosterone acetate used in this study was provided through the courtesy of Doctor E. Oppenheimer, Ciba Pharmaceutical Products, Inc., Summit, N. J.

evidence of functional impairment was reported by Novak (16) following adrenalectomy in rats. This was associated with some reduction in the size of the testes. Freed, Brownfield and Evans (17) reported reduction in testicular weight with tubular degeneration in adrenalectomized rats. Jaffe and Marine (18), in rabbits, found only a small percentage of cases of tubular degeneration in the testes. Shumacker and Firor (19) observed no very consistent or marked changes in testis weights or histology in adrenalectomized rats, but did find a definite reduction in the size of prostates and seminal vesicles. From these studies it is not clear to what extent the effects on the prostate and vesicles are due to adrenal insufficiency or are secondary to impairment in output of testicular hormone. Evidence of the importance of the pituitary was presented (17, 19). The variability of the findings as to tubular degeneration of the testes may be due to varying amounts of cortical accessory tissue which were insufficient to maintain a normal condition but sufficient to affect the testicular condition.

Of more interest in the present connection are the effects of adrenalectomy on seminal vesicles and prostates in castrated animals. Such studies have been reported by the author in preliminary form for mice (20) and rats (21) and by Burrill and Greene for rats (22, 23). Since the findings in the two species differ somewhat, the results obtained in mice will be discussed in a subsequent communication.

Burrill and Greene (22, 23) reported a considerable reduction in the size of the prostate in adrenalectomized castrated immature rats as compared to control castrates, with corresponding evidence of histological regression after adrenalectomy. Since the prostates were smaller not only absolutely but also in relation to body weight, it was concluded that the reduction was in part at least the result of a specific inhibition of the organ due to the absence of adrenal androgens. A specific reduction in prostate size could be considered as due to lack of androgen if an androgen is regarded simply as a substance which causes growth of the prostate. However, the findings might also be accounted for if the gland were particularly sensitive to the metabolic deficiencies produced by adrenalectomy. We have no reason to assume that all tissues need suffer the same degree of growth inhibition in adrenal insufficiency, and, as noted above, degenerative changes have been observed in a variety of tissues after adrenalectomy. Accordingly it appeared desirable to extend these observations to include a study of the prostates of adrenalectomized castrates in which body growth was maintained as nearly normal as possible by cortical substitution therapy.

Greene and Burrill (24) later reported that desoxycorticosterone did not prevent the prostatic atrophy in three-week-old adrenalectomized castrates, but they failed to give data on the body growth of their animals, and they did not use their results with desoxycorticosterone as evidence for the point at issue.

Gersh and Grollman (25), on the other hand, reported that the prostates of young castrated adrenalectomized rats treated with a "non-androgenic" extract of the cortex were not appreciably different from the glands of castrates, and hence concluded that the adrenal does not normally secrete physiologically detectable amounts of androgens. They reported similar conclusions for mice. They stated that their animals were maintained "in good condition" on the cortical extract but did not give quantitative comparisons between the growth of normal animals and the treated adrenalectomized group. The total number of rats used was small and the authors were apparently not aware of the extreme shortness of the period in rats in which the anomalous postcastration maintenance of the prostate is well developed.

In view of these considerations, the present studies of the effects of adrenalectomy and of desoxycorticosterone acetate substitution therapy is castrated rats have been made.

EXPERIMENTAL

Rats were castrated at 17 days of age, and adrenalectomized at 20 days. Half of those which were adrenalectomized were given subcutaneous pellets of desoxycorticosterone acetate. Litters were divided so that each contained 1 or more each of *a*) castrates, *b*) adrenalectomized castrates and *c*) treated adrenalectomized castrates.

TABLE 1. EFFECT OF ADRENALECTOMY AND DESOXYCORTICOSTERONE ON GROWTH OF BODY AND OF VENTRAL PROSTATE IN RATS

C, castrate controls; A C, adrenalectomized castrates; A C D, adrenalectomized castrates treated with desoxycorticosterone acetate.

	C	A C	A C D
Av. body wt. at adrenalectomy, gm.	37.3	37.6	37.6
No. of rats	11	11	13
Wt. increase, % of original wt.	62.4	22.4	57.7
Wt. increase, % of castrate	100	36	93
Prostate wt./body wt. $\times 10^6$	192	101.3	95.0
Prostate wt./body wt., % of castrate	100	53	50

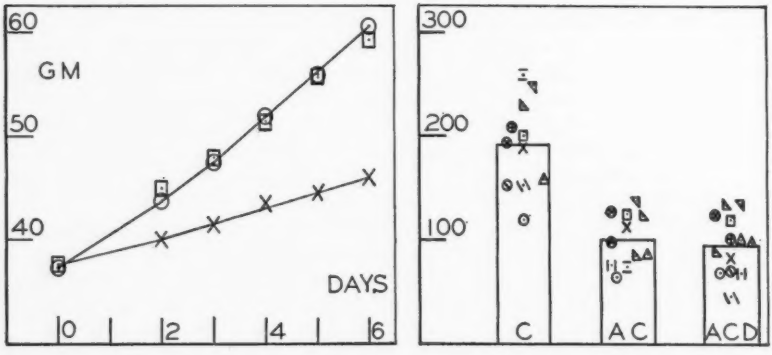

Fig. 1. EFFECT OF ADRENALECTOMY AND DESOXYCORTICOSTERONE ACETATE SUBSTITUTION THERAPY ON BODY GROWTH OF YOUNG RATS. Vertical axis, wt. in gm.; horizontal axis, days after adrenalectomy. Animals had been castrated 3 days previous to adrenalectomy. *Circles*, castrates; *crosses*, adrenalectomized castrates, *rectangles*, adrenalectomized castrates with desoxycorticosterone. The line of normal growth is drawn through the values for the castrates. Fig. 2. SCATTER GRAPH OF PROSTATE SIZE AS INFLUENCED BY ADRENALECTOMY. Vertical axis: ventral prostate body wt. ratio $\times 10^6$. Experimental groups are arranged along the horizontal axis: C, castrates; AC, adrenalectomized castrates; ACD, adrenalectomized castrates treated with desoxycorticosterone acetate. Average values are indicated by the height of the columns. Litter mates are indicated by similar symbols around the individual points.

They were returned to their mothers a few hours after the operations. Growth was followed by daily weighings, and they were killed with ether at 26 days of age. When rats of this age are allowed to nurse it was found that the average survival time after adrenalectomy, determined on littermate females, was 8 days, hence it was unnecessary to use other ameliorative treatment to prolong the survival time of the untreated adrenalectomized castrates. Animals were fed Purina chow and water.

Results are presented in table 1, and figure 1. It was found that growth of the untreated adrenalectomized castrates was consistently much less than that of the castrates. The treated adrenalectomized castrates grew well but after 3 or 4 days usually showed a tendency to lag behind the growth of the castrates to a small de-

gree. A group of 7 adrenalectomized females with pellets were compared with normal female littermates, and approximately the same lag in growth was observed.

The pellets were made by pounding crystals of desoxycorticosterone acetate in a cylindrical mold. They weighed 23 to 30 mg. initially, and lost weight at the rate of 0.2 to 0.4 mg. per day per rat. The pellets did not become fixed in position by a thick vascularized connective tissue capsule, as in mice, but were covered by a very slight amount of connective tissue. They moved freely in the subcutaneous space. In an effort to maintain growth completely equivalent to that of the castrate controls more desoxycorticosterone acetate was given both by oil injections and by implanting additional numbers of pellets. Daily injections of 1 mg. in corn oil in addition to a pellet, produced no consistent appreciable improvement in growth, nor did the implantation of 2 or 3 pellets produce greater growth. It may be concluded that the dosage of desoxycorticosterone acetate is not the limiting factor, but that presumably some other component of the cortical hormone complex is required. However as shown in

TABLE 2. EFFECT OF ADRENALECTOMY AND DESOXYCORTICOSTERONE ON GROWTH OF BODY AND OF VENTRAL PROSTATE IN RATS RECEIVING 2% NaCl

C, castrate controls; A C, adrenalectomized castrates; A C D, adrenalectomized castrates treated with desoxycorticosterone acetate.

	C	A C	A C D
Av. body wt. at adrenalectomy, *gm.*	36.8	35.7	35.6
No. of rats	7	12	11
Wt. increase, % of original wt.	34	17	21
Wt. increase, % of castrate	100	50	62
Prostate wt./body wt. $\times 10^6$	236	100	115
Prostate wt./body wt., % of castrate	100	42	49

table 1 an average weight increment of 93% of that of the castrates was attained by the treated adrenalectomized castrates during the short experimental interval of 6 days. This observation of a partial deficiency in the growth-maintaining properties of desoxycorticosterone acetate should be compared with the findings of Wells and Kendall (26) who found that in normal rats desoxycorticosterone produced growth slightly greater than that of the normal. The effects of desoxycorticosterone in the presence of the adrenals might be expected to differ from its effects in adrenal insufficiency.

In another series of rats treatment with 2% NaCl in place of drinking water did not improve the growth of the adrenalectomized animals. The data regarding the salt treated rats are given in table 2. By comparison of tables 1 and 2 it may be seen that the effect of salt treatment was to reduce the per cent weight increase during the 6-day experimental period. In the case of the castrate controls the weight increment was decreased from 62 to 34%; in the adrenalectomized castrates from 22 to 17%; and in the adrenalectomized castrates receiving desoxycorticosterone from 58 to 21%. Some litters were given 2% NaCl throughout the 6-day experimental period, although they showed evidence of very abnormal thirst and dehydration. Other litters were returned to water or given 1% NaCl before the end of the 6-day period. They responded to the decreased NaCl intake by improved growth. All data for the NaCl treated rats are combined in table 2, so that the effect of the salt treatment for the full 6-day period in all cases would probably show a somewhat greater interference with growth than is indicated in the table. In any case the toxicity of 2% NaCl was manifest in both the castrate controls and in the adrenalectomized rats receiving desoxycorticosterone, but to a greater degree in the latter. Such a concen-

3
4
5
6
7
8

tration of NaCl would be expected to put a considerable strain on the kidneys of a normal animal.

The state of the prostate in the 2 series was similar, but since general body growth was so inferior in the NaCl series, the chief interest attaches to the glands of the series which did not receive NaCl. Unless otherwise specified, findings refer to the state of the glands in the series without NaCl.

At autopsy the ventral prostates were dissected out, fixed in Bouin's fluid for 12 to 18 hours, washed in water for 1–2 hours, dried in a uniform manner on filter paper for 30 seconds, and weighed. The ventral prostate lobes are discrete, readily isolated structures. Dissection was facilitated by hardening with a drop of Bouin's fluid after the lobes had been isolated and separated from fat. The technic of drying and weighing was reproducible with a probable error of ± 0.1 mg. After weighing, the glands were embedded in paraffin, sectioned at 10μ and stained in hematoxylin.

The average prostate weights for animals not receiving NaCl in water are given in table 1, as % of body weight, and the distribution of the individual values is shown in figure 2. It will be seen that the percentage weights of the prostates in the treated adrenalectomized castrates were very slightly less than in the untreated adrenalectomized castrates, hence the prostate probably shares in the general body growth for which the cortical hormone is essential. However in terms of % of body weight there was no stimulation of the prostate in the presence of the desoxycorticosterone. On the contrary, there was manifest a 50% reduction in the size of the prostate after adrenalectomy. The average figures for prostate size of 53% of the control weight in the untreated adrenalectomized rats, and of 42% in the NaCl series agree satisfactorily with Burrill and Greene's figure of 47% (23).

In addition to this confirmation of Burrill and Greene's findings as to the effect of adrenalectomy on the prostate, we present the further fact that this suppression of prostate growth occurred even when systemic body growth was maintained at 93% of that of the castrate controls by substitution of desoxycorticosterone acetate treatment for functioning adrenal cortex.

Histological examination of the prostates showed definite differences between the castrates and the adrenalectomized castrates. The histological state of the glands was in general proportional to their percentage weights. Typical areas are illustrated in figures 3 to 8.

A systematic microscopic study was made of sections taken at regular intervals from complete serial sections of the glands, cut at 10μ and stained in Ehrlich's hematoxylin, with subsequent differentiation in saturated Li_2CO_3. In the castrate controls the epithelium was generally higher in the peripheral acini, and lower in the central acini which usually appeared to be storage regions with cuboidal epithelium. Consequently a general survey of all parts of the gland was necessary to evaluate its condition.

The prostates from the castrated animals were characterized by large acini with considerable areas of well developed high columnar epithelium. The light areas in-

PLATE I. Fig. 3 TO 8. PHOTOGRAPHS OF SECTIONS OF RAT PROSTATES. Fixation, Bouin's fluid, stain, Ehrlich's hematoxylin. All animals were killed at 26 days of age. Fig. 3. Prostate of castrated rat, fairly large alveoli and columnar epithelium. Fig. 3, 4 and 5 are from litter mates. ×100. Fig. 4. Prostate of adrenalectomized castrate. Note reduction in size of alveoli and cuboidal epithelium. ×100. Fig. 5. Prostate of adrenalectomized castrate carrying 3 pellets of desoxycorticosterone. Note similarity to gland in fig. 4. ×100. Fig. 6. Tall columnar epithelium from prostate of castrate. Note presence of light area midway between nucleus and lumen end of cell. Fig. 6, 7 and 8 are from litter mates. ×750. Fig. 7. An area of the best developed epithelium from the prostate of an adrenalectomized castrate. Note reduction in cell height in comparison with fig. 6, and the absence of light areas. Most of the epithelium in glands from these animals was cuboidal. ×750. Fig. 8. An area of the best developed epithelium from the prostate of an adrenalectomized castrate treated with desoxycorticosterone. Note similarity to fig. 7. ×750.

dicative of the presence of androgens (5) were present in the cells of some acini in all animals, but there was considerable variation in the extent of epithelium with light areas. In some cases there were only a few acini having cells with light areas; these were faint and ill-defined in comparison to their condition in those glands in which they were in their best developed state. In some glands there were areas in which some of the peripheral secretory epithelium was undergoing degeneration.

In the adrenalectomized castrates the state of the prostates was markedly inferior to that in the castrates. The acini were much smaller. There was no well developed high columnar epithelium, and there were no light areas in any acini. The epithelium varied from low cuboidal (with, in some cases, apparently beginning disorganization of the acinar structure and migration of cells into subjacent connective tissue), to medium low columnar (often with the appearance of pseudo-stratification due to crowding of the involuting cells). The cells showed evidence of shrinkage, and irregular vacuolated areas appeared frequently. In general the prostates of the adrenalectomized castrates were in a uniform state of marked degeneration.

In the group of adrenalectomized castrates carrying pellets of desoxycorticosterone acetate there was in 13 animals no evidence of any improvement in condition in comparison to that of the untreated adrenalectomized castrates. In one case the gland weight was equal to that of the control and there was only partial histological regression. This animal also showed no interference with its normal growth; it was excluded from the general average on the grounds that accessory adrenal tissue had probably been present. With this exception, it was found that although there was considerable variation among different litters in the absolute levels, both the weights and the histological states showed consistent differences when animals from the different groups within the same litter were compared. Thus the prostates of the adrenalectomized castrates showed no detectable differences from the adrenalectomized castrates treated with desoxycorticosterone. There was a marked effect of adrenalectomy in reducing the anomalous partial maintenance of the prostate after castration at this age.

The histological picture in the prostates was not appreciably affected by the 2% NaCl treatment. The prostates of castrates which had received NaCl showed a degree of maintenance of the condition of the epithelium similar to that of the glands of those on water; the glands of the adrenalectomized animals were uniformly degenerate. The lack of difference between the prostates in the series which grew well and in the series in which growth was partially inhibited by 2% NaCl is further evidence that the prostate stimulation in the presence of the adrenal is, in rats, a specific effect distinct from general body growth.

DISCUSSION

Desoxycorticosterone is satisfactory in replacing the adrenal cortex insofar as it maintains young animals in a state which permits practically normal growth. Since the prostate does not exhibit normal castrate development in the adrenalectomized castrate maintained on desoxycorticosterone the most probable conclusion seems to be that an adrenal androgen is lacking. Although it would be desirable to supplement the present observations by a study of the prostate in castrated adrenalectomized rats maintained on other cortical sterols when these become more freely available in pure form, it seems improbable that the prostate development should be secondary to a specific effect of a cortical sterol on, for instance, carbohydrate metabolism. It is more probable that we are dealing with a capacity of the cortical cells to produce an androgen, dissociable from sterols with predominant effects on general metabolism.

It is concluded that the adrenal is capable of producing physiologically appreciable amounts of material which cause some stimulation of the prostate in young castrated rats. The type of stimulation is characteristic of that produced by androgens (5). However, it must be emphasized that the period of this adrenal activity is normally very short. Price (2) showed that the anomalous postcastration maintenance of the rat prostate was entirely over by 50 days of age, and had materially subsided considerably before this age. These time relationships were confirmed both by Howard (3) and by Burrill and Greene (23).

Not only is the period of this prostate development very brief, but the gland is never stimulated to the point of the production of the abundant secretion characteristic of the mature intact male. At present we have no evidence as to whether this is due only to the brief period and small amount of adrenal androgen production, or to some qualitative difference between the mode of functioning of adrenal and testicular androgens. Hence it may be preferable to speak of the andromimetic rather than androgenic activity of the adrenal cortex.

It has been reported that considerable quantities of androgens are present in the urine of adult human castrates (27), and it has been supposed that these may arise from the adrenal. If this is shown to be their source it would seem that there may be considerable differences between man and rat in this respect. However, this might well be the case in view of the rather large species differences in the amounts of sex hormones excreted under various conditions.

The finding of a brief phase of physiologically appreciable androgen production by the rat adrenal may be of general biological significance in several ways. It is a specific example of a physiological activity which appears to be accentuated in certain tumors of the gland. However, its significance in relation to the early development of the reproductive system remains to be examined in embryological studies. Its chief interest may lie in its being an example of a modulation in an endocrine function during the life span of the individual. In connection with the problems of aging, the life history of the endocrine functions may be of importance.

SUMMARY

Prostate weights are reduced by 50% following adrenalectomy at 3 weeks of age in castrated rats. The weight loss is associated with histological degeneration.

The prostate degeneration is not prevented when adrenalectomized castrated rats are maintained on desoxycorticosterone with a body growth rate 93% of that of castrated controls.

It is concluded that the rat adrenal is capable of secreting physiologically appreciable amounts of androgens during a certain phase in the development of the sexually immature animal.

REFERENCES

1. Reichstein, T.: *Helvetica Chim. Acta* 19: 223. 1936.
2. Price, D.: *Am. J. Anat.* 60: 79. 1936.
3. Howard, E.: *Am. J. Anat.* 62: 351. 1938.
4. Howard, E.: *Am. J. Anat.* 65: 105. 1939.
5. Moore, C. R., D. Price and T. F. Gallagher: *Am. J. Anat.* 45: 71. 1930.
6. Moore, C. R., W. Hughes and T. F. Gallagher: *Am. J. Anat.* 45: 109. 1930.
7. Howard, E.: *Am. J. Physiol.* 119: P339. 1937.
8. MacMahon, H. E., and R. L. Zwemer: *Amer. J. Path.* 5: 491. 1929.
9. Banting, F. G., and S. Gairns: *Am. J. Physiol.* 77: 100. 1926.
10. Hartman, F. A., C. G. MacArthur, F. D. Gunn, W. E. Hartman and J. J. MacDonald: *Am. J. Physiol.* 81: 244. 1927.
11. Swingle, W. W.: *Am. J. Physiol.* 79: 666. 1927.
12. Marine, D.: *Arch. Path.* 6: 169, Proc. 1928.

13. Simpson, S. L., and V. Korenchevsky: *J. Path. and Bact.* 40: 483. 1935.
14. Gersh, I., and A. Grollman: *Am. J. Physiol.* 125: 66. 1939.
15. Marshall, E. K., Jr., and D. M. Davis: *J. Pharmacol. Exper. Therap.* 8: 525. 1916.
16. Novak, J.: *Arch. f. Gynak.* 101: 36. 1913.
17. Freed, S. C., B. Brownfield and H. M. Evans: *Proc. Soc. Exper. Biol. & Med.* 29: 1. 1931.
18. Jaffe, H. L., and D. Marine: *J. Exper. Med.* 38: 107. 1923.
19. Schumacker, H. B., and W. M. Firor: *Endocrinology* 18: 676. 1934.
20. Howard, E.: *Am. J. Physiol.* 126: P539. 1939.
21. Howard, E.: *Am. J. Physiol.* 133: P336. 1941.
22. Burrill, M. W., and R. R. Greene: *Proc. Soc. Exper. Biol. & Med.* 40: 327. 1939.
23. Burrill, M. W., and R. R. Greene: *Endocrinology* 26: 645. 1940.
24. Greene, R. R., and M. W. Burrill: *Proc. Soc. Exper. Biol. & Med.* 43: 382. 1940.
25. Gersh, I., and A. Grollman: *Am. J. Physiol.* 126: 368. 1939.
26. Wells, B. B., and E. C. Kendall: *Proc. Staff Meet. Mayo Clinic* 15: 133. 1940.
27. Callow, N. H., R. K. Callow and C. W. Emmens: *J. Endocrinology* 2: 88. 1940.

MOLECULAR-EQUIVALENTS VERSUS WEIGHT-EQUIVALENTS IN THE POTENCY OF ANDROGENIC SUBSTANCES

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THE RELATIVE POTENCIES OF DIFFERENT ANDROGENS have commonly been determined by comparing the growth responses of end organs (capon's comb, prostate and seminal vesicles of rodents) to equal weights of the substances expressed in milligrams or fractions thereof. Such comparisons neglect the fact that some androgens contain non-active moieties and thus equal weights of different androgens may vary in the amounts of active hormonal substance present. For example, in testosterone propionate, 16% of the weight of the molecule consists of the aliphatic acid radical (propionic acid) of this ester which is androgenically inactive. Testosterone should therefore be compared with testosterone propionate on the basis of equivalent molecular amounts of the two substances, in order to allow for the different amount of active material in the two molecules.

It is generally accepted, although very often neglected, that the biological, toxicological or pharmacological activities of various drugs should be compared on a molecular weight basis. Such a procedure seems also necessary in the case of the hormones, particularly since the esterified forms are widely used because of their prolonged action. The form assumed by the active substance after the administration of sex hormones is not known. The form in which esterified hormones are absorbed is also unknown. There is, however, sufficient evidence to believe that they do not reach the end organs as esters. The acid radicals in such an instance, therefore, is added weight, although desirable because of its function in prolonging activity presumably through delayed absorption from the site of administration. The esterified form, however, should not be penalized in comparisons with the free form because of this added androgenically inactive component.

The present study demonstrates that allowance for the weight of the acid radical is of the utmost importance in comparing the percutaneous potency of testosterone with that of testosterone propionate.

PROCEDURE

One hundred male rats, 150 ± 10 gm., were castrated and divided into 5 groups, each containing 20 animals. The hair of the back, over an area of 15 to 20 sq. cm. was removed with scissors and reclipped whenever necessary during the course of the experiment. The ointments containing the androgens were applied to this area. The concentrations of the testosterone and testosterone propionate[1] in the ointments were such that the desired dosages could be applied in 0.1 cc. of ointment in all groups.

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[1] Perandren—Ciba, Summit, N. J.

Accurate measurement of this amount of ointment was facilitated by applying it directly from a tuberculin syringe. The barrel of the syringe was used to rub the ointment into the skin. The particular ointment used contained:

tragacanth powder	4	gm.%
alcohol	20	cc.
hydrogenated cottonseed oil	4.99	cc.
nipagin	0.2	gm.
distilled water	70.6	cc.

In all cases treatment was administered daily, beginning the day after castration. In 3 groups the treatment was continued for 21 days and in 2 groups for 10 days.

TABLE 1. COMPARISON OF PERCUTANEOUS POTENCY OF TESTOSTERONE AND TESTOSTERONE PROPIONATE IN MALE RATS

Gr. No.	Substance	Daily Dose	Length of Treatment	Prostate Weight			Seminal Vesicle Weight		
				mean (σ)	Crit. Ratio[1]		mean (σ)	Crit. Ratio	
					T.P. 0.2	T.P. 0.239		T.P. 0.2	T.P. 0.239
		mg.	*days*	*mg.*			*mg.*		
1	Test. Prop.	0.2	21	72.5 (4.68)		8.19	62.5 (5.28)		7.18
2	Testoster.	0.2	21	102.0 (7.04)	3.45	2.30	92.0 (12.2)	2.22	1.57
3	Test. Prop.	0.239	21	120.0 (3.42)			126.0 (17.99)		
4	Testoster.	0.2	10	88.0 (5.60)		0.71	79.0 (9.13)		0.78
5	Test. Prop.	0.239	10	96.0 (9.95)			91.0 (12.34)		

[1] Critical ratio $= \frac{\text{obtained difference}}{\sqrt{(\sigma_{m1})^2+(\sigma_{m2})^2}}$

Significant critical ratios are underlined.

σ = standard deviation from the mean $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$

The animals were killed on the day following the last treatment. The prostates and seminal vesicles were carefully removed under a dissecting microscope and immediately weighed.

The mean weights and standard deviations from the mean for the several groups were calculated and the statistical significance of these figures evaluated. Since there were 20 animals in each group, critical ratios of 2.86 or more were considered significant.

RESULTS

The results are summarized in table 1. At autopsy the mean body weights of the different groups did not vary appreciably (186 to 194 gm.) and the differences between groups were not significant. Data on body weights are therefore not tabulated.

Comparison of weight-equivalents. When testosterone and testosterone propionate were given in equal milligram amounts of 0.2 mg. daily (*groups* 1 and 2), testosterone produced ventral prostates which were significantly larger than those produced by testosterone propionate (critical ratio 3.45). The seminal vesicles of the testosterone group were likewise larger, but not significantly so (critical ratio 2.22).

Comparison of molecular-equivalents. To compensate for the weight of the androgenically inert portion of the testosterone propionate molecule, the daily dose of this substance was increased to 0.239 mg. (*groups* 3 and 5). As will be seen in table 1,

testosterone propionate at this dose was no longer inferior to testosterone. In both the 21-day and the 10-day treatment groups, the mean prostate and seminal vesicle weights were slightly larger following the administration of testosterone propionate than those following non-esterified testosterone. The differences, however, were not significant. In contrast to the results obtained on the equivalent weight basis, these data demonstrate that, molecule for molecule, testosterone propionate is just as potent as testosterone when administered percutaneously in this ointment.

DISCUSSION

It should be stressed that conclusions based on these data apply only to the comparative potency of testosterone and testosterone propionate administered *in the ointment which we have used.* We have certain data at hand which indicate that the type of ointment used may alter the percutaneous potencies of testosterone, testosterone propionate and methyl testosterone and may even, in some instances, reverse their relative potencies. The vehicle appears to have an important effect on the absorption of these compounds. It follows, therefore, that in further experimental or even clinical work on this subject, conclusions regarding relative potencies of androgens should be restricted to the specific conditions under which they are used.

Moore, Lamar and Beck (1) and some of the present authors (2) have previously compared equal milligram amounts of testosterone and testosterone propionate and have concluded that, administered percutaneously, testosterone is more effective than testosterone propionate. Such conclusions are not valid as generalizations, but must be qualified since they apply only to comparisons of equal weights of the hormones and are limited to the particular vehicles used in the studies.

It has been stated repeatedly that testosterone propionate, when given subcutaneously, is far superior in potency to non-esterified testosterone. These comparisons also have been made only on the basis of weight equivalents. If due allowance were made for the inactive part of the molecule and the two substances were compared on the basis of molecular equivalents, the order of potency of these two androgens would probably not be changed. It might be of theoretical interest, however, to note how much greater would be the potency of testosterone propionate if this consideration were applied.

SUMMARY AND CONCLUSIONS

Testosterone was percutaneously more effective than equal milligram amounts of testosterone propionate. There was, however, no significant difference in the effectiveness of the two when equal molecular amounts were administered. It was concluded, therefore, that in the ointment used in this study, there was no difference, molecule for molecule, in the percutaneous effectiveness of testosterone and testosterone propionate.

REFERENCES

1. Moore, C. R., J. K. Lamar and N. Beck: *J. Amer. Med. Assoc.* 111: 11. 1938.
2. Nelson, D., R. R. Greene and J. A. Wells: *Endocrinology* 26: 651. 1940.

EFFECT OF TESTOSTERONE PROPIONATE ON VARIOUS RESPONSIVE ORGANS OF IMMATURE RATS[1]

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ONE OF THE OBVIOUS approaches to the problem of differentiating and of assaying sex hormones is to determine the characteristic curves produced by each in a variety of sensitive tissues which may be designated as target organs in test animals. The absolute effects and, presumably, the relationships between the curves may be expected to vary somewhat from colony to colony even when all due care is paid to accuracy of autopsy procedures. Hence individual curves must be worked out for the animal colony in each institution using the method. The results shown in the accompanying graph have been obtained in our rat colony in four-day assays of testosterone propionate.[2]

METHOD

A Wistar strain of albino rats bred in our own colony was used exclusively. The initial age and weight ranges of the subjects were limited from 20 to 25 days and from 30 to 35 gm. respectively. Any test animal was discarded if, before the end of the test, its weight went beyond the extremes of the range set above.

Only those litters were used in which there were at least 4 eligible offspring of the same sex. Of these, one litter-mate was used as a 'normal control,' the second as an 'oil control,' the third was given a low-range dose, and the fourth a high-range dose of testosterone propionate. This arrangement served to control variations of response within litters.

Each test animal was injected daily, at the same hour, for each of 3 consecutive days and was autopsied 24 hours after the third injection. The individual doses of the androgen were: 0.5, 1.0, 2.0, 4.0, 8.0, and 10.0 mg. respectively; and, in the case of female rats, two additional doses of 0.20 and 0.75 mg. were used. The doses were diluted with pure sesame oil so that each was contained in either 0.05, 0.075, 0.10, 0.20, 0.40, or 0.50 cc. These amounts of oil were utilized in the 'oil-control' series. All injections were made in standard sequence intramuscularly or intraperitoneally; the same injection site was never used twice in a single animal.

At the time of autopsy, all animals of each litter were dissected and the target organs removed. These were immediately trimmed of their accessory tissue, using a Beebe binocular loupe. The organs were then gently blotted and weighed on a torsion balance to the nearest milligram, before drying could occur.

The following items were recorded: total body weight just prior to autopsy; the weight of the thymus, all lobes of the prostate together with both lobes of the seminal vesicles, both testes, both capita epididymi, the uterus, and both ovaries. The means

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(M) and standard deviations (S.D.) for the total body weight and each organ weight per dose of testosterone propionate are listed in table 1. The organ weights were then reduced to percentages of the body weight.

A minimum of 20 surviving test animals of each sex, per dose, was accumulated. The number of 'normal controls' being ¼ of the total nomber of each litter, of course greatly exceeded twenty. It was found that the oil injections had no significant influence on the organ weights hence only the 'normal control' averages were used as a basis for further consideration of the data.

The final average gland-to-body-weight ratios of the treated rats were further

TABLE 1. MEANS AND STANDARD DEVIATIONS OF WEIGHTS OF ORGANS IN RESPONSE TO VARIOUS DOSES OF TESTOSTERONE PROPIONATE

	Normal	Testosterone propionate, dosage, mg.							
		0.2	0.5	0.75	1.0	2.0	4.0	8.0	10.0
Autopsy Body Wt., ♀, gm.	34.5 ±2.4	35.4 ±2.8	35.3 ±2.8	33.9 ±1.9	34.1 ±2.9	34.6 ±2.5	34.9 ±2.4	34.4 ±2.7	34.7 ±2.4
Uterus, mg.	19.4 ±6.1	75.6 ±8.4	81.7 ±9.6	83.8 ±12.1	85.2 ±15.3	92.3 ±12.1	101.4 ±13.3	105.8 ±11.0	107.6 ±10.04
Ovaries, mg.	10.1 ±2.4	10.6 ±1.6	10.6 ±2.4	10.2 ±1.8	9.8 ±2.3	11.1 ±2.1	11.9 ±3.3	10.7 ±3.0	11.3 ±2.0
Thymus, ♀, mg.	110.9 ±25.7	106.3 ±28.0	109.9 ±30.8	97.0 ±18.9	92.4 ±23.7	92.0 ±19.3	92.8 ±15.6	83.7 ±20.6	88.6 ±17.6
Autopsy Body Wt., ♂, gm.	34.9 ±2.4	—	35.7 ±3.1	—	34.8 ±2.7	35.5 ±3.1	35.7 ±2.9	35.4 ±3.1	34.0 ±3.2
S. V./Pros., mg.	48.2 ±12.5	—	79.2 ±14.7	—	84.4 ±13.6	86.5 ±14.4	88.1 ±16.4	89.5 ±15.7	85.1 ±15.4
Caput Epidid., mg.	10.5 ±2.3	—	17.2 ±3.0	—	17.8 ±3.1	17.9 ±2.6	17.5 ±3.9	18.4 ±3.8	17.3 ±3.0
Testes, mg.	163.5 ±26.1	—	231.7 ±39.6	—	227.0 ±32.5	237.1 ±29.6	233.0 ±36.7	226.1 ±52.1	227.9 ±38.6
Thymus, ♂, mg.	105.8 ±25.3	—	97.9 ±17.8	—	94.1 ±19.7	93.0 ±20.9	84.9 ±16.8	86.9 ±22.8	81.6 ±26.3

reduced to positive or negative percentile deviations from the values of the 'normal control' series. These deviations are plotted on the accompanying graph (fig. 1).

RESULTS

The uterus proved to be the most sensitive organ-indicator, a fact which confirms the work of Sevringhaus (1) and others. As the daily dosage increased from 0.2 to 10.0 mg., the percentage increase rose from 284 to 459, with a suggestion of flattening off at 10 mg.

The seminal vesicle-prostate combination was found to be the next most sensitive organ-indicator but it was much less so than the uterus. The increase ranged from 61.6 to 81.9%. The curve indicates a gradual lessening of response beginning at the 2.0 mg. dose. The 8.0 mg. dose gave the greatest response, an 86.2% increase, at the 10.0mg dose the increase dropped to 81.9%.

The capita epididymi almost paralleled the response of the seminal vesicle-prostate

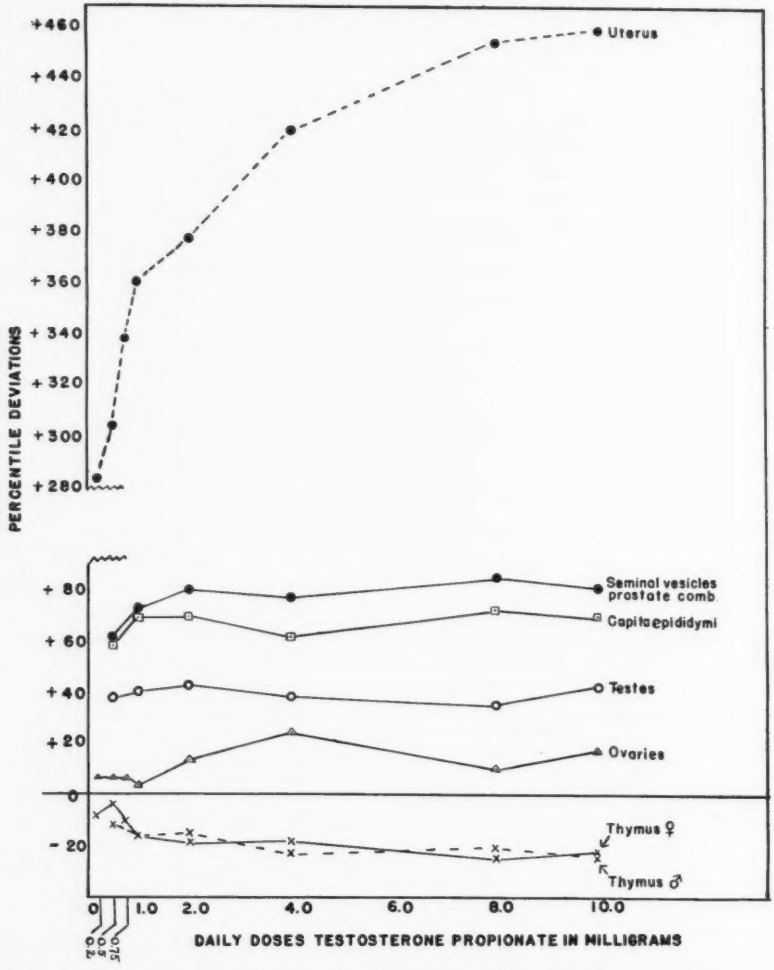

Fig. 1. Percentile deviations of averages of organ-body weight ratios of experimental animals from those of the normal controls.

combination but at a slightly lower level, ranging from 60.0 to 73.4%. Again there is a lessening in response at the 10.0 mg. dose, the deviation being only 70%.

The testes ranged from 38.1 to 43.0% above that of the normal controls.

The ovaries proved to be the least sensitive organs, the increase above that of the normal controls ranging from 3.6 to 25.0%. The latter value was obtained with the 4.0 mg. dose after which the response lessened to a 10.7 and 17.9% increase with the 8.0 and 10.0 mg. dose respectively.

The thymus showed a steady inhibition in growth as the dose of testosterone propionate was increased. This negative response ranged from −2.8 to −23.6%, the latter figure being obtained with the 8.0 mg. dose in the female rats. Separate

curves of thymic response are drawn for each sex and the curves indicate that the inhibition of growth is practically the same in both. This finding confirms that reported by Persike (2).

The body-weights were not sensibly influenced during the brief treatment period by either the androgen or the sesame oil.

It is to be noted that in those animals receiving the larger volumes of sesame oil, that is the 4.0, 8.0, and 10.0 mg. doses of testosterone propionate which were given in 0.4, 0.4, and 0.5 cc. of sesame oil repectively, that there was always one area of incompletely absorbed oil found at autopsy. However, this was true also in those 'oil control' animals receiving equivalent amounts of pure sesame oil. The areas of incomplete oil absorption were aways at the site of the third injection, given 24 hours before autopsy. This raises the questions *a*) was any of the testosterone propionate absorbed from the third injection in the groups receiving the three largest doses; *b*) if any was absorbed, was the rate of absorption constant or inconstant from animal to animal: or *c*) was all of the testosterone absorbed despite the incomplete absorption of the sesame oil? It is obvious that no factual statement can be made regarding this but there is some indirect evidence in that the standard deviations of the values from the animals which received the larger doses do not vary significantly from the standard deviations of the values from the animals receiving the smaller doses. It may be reasoned, therefore, that the amounts of testosterone not absorbed from the larger doses was, at least, fairly constant. It is, however, much better to avoid areas of incomplete-oil-absorption and this can be accomplished easily by administering the doses of the hormone in 0.2 cc. or less of sesame oil.

SUMMARY

Testosterone propionate was given to groups of young male and young female rats in individual doses of 0.50, 1.0, 2.0, 4.0, 8.0, and 10.0 mg. respectively. The female rats were given two additional doses of 0.20 and 0.75 mg. respectively. The selected dose for each test animal was given on each of 3 successive days and the animals, including the litter-mate 'controls,' were autopsied 24 hours after the third injection.

The effects on several target organs, both testes, both capita epididymi, both seminal vesicles together with all lobes of the prostate, both ovaries, the uterus, and thymus, were determined.

It was found that the uterus was the most sensitive target organ, the seminal vesicle-prostate combination next, and then the caput epididymus and testis; the ovary was the least responsive. The growth of the thymus was inhibited.

REFERENCES

1. Sevringhous, E. L., C. G. Hiller, H. Lauson and J. B. Golden: *J. Biol. Chem.* 123: 108. 1938.
2. Persike, E. C.: *Am. J. Physiol.* 130: 384. 1940.

Sometime after the paper was submitted for publication, 'Fluhman Indices' (*Endocrinology* 18: 705. 1934) were determined on histological sections of the vagina. For the dose-range used, these were 0, 2, 2, 2, 2, 3, 4, and 4 respectively, 0 representing the index of normal controls. This indicates that vaginal epithelium is moderately sensitive to testosterone stimulation.

CREATINE-CREATININE METABOLISM AND THE HORMONES[1]

VII. FURTHER OBSERVATIONS UPON THE BIOLOGICAL RELATIONSHIP BETWEEN CREATINE AND CREATININE IN RELATION TO WATER, SALT AND PHOSPHATE METABOLISM IN NORMAL AND CASTRATE RATS

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IN ONE OF OUR RECENT PUBLICATIONS upon this subject it was shown that in both rat and man injected or ingested creatine was not transformed into creatinine but caused its retention instead (1). Several recent investigations have denied any biologic relationship between creatine and creatinine and our recent results agree with this conclusion. Furthermore, it was shown that creatinine, under similar experimental conditions, caused an increase in muscle creatine and stimulated enormously the excretion of both creatine and creatinine in the urine. Increases of from 5 to 21 gm. of creatine and of 2 to 10 gm. of creatinine were excreted in man after ingestion of only 1 gm. of creatinine by mouth. By the use of the specific creatinine enzyme of Miller and Dubos (2) it was shown that these enormous increases in the color of the Jaffe reaction with alkaline picrate were due to creatinine alone.

If the above observations are correct it is obvious that creatinine may be hydrated to creatine in the body. Although this observation was made many years ago it has never been generally accepted (1). From the chemical point of view creatinine is transformed into creatine at an alkaline pH and the reverse change occurs at an acid pH. Brazda, Espenan and Beard (3) observed that, at a pH range from 6.7 to 7.6, four times as much creatinine was transformed into creatine in two weeks as compared to the reverse process. The average amount of creatine formed from 100 mg. of creatinine in 100 cc. of solution was 36 mg. in 28 days. Beard and Jacob (1) injected from 10 to 200 mg. of creatinine into 10 groups of young white rats. The average extra creatine excretion in these rats was 37 mg. at the end of 21 days. Hence an excellent agreement between the *in vitro* and *in vivo* formation of creatine is afforded by our studies.

The results reported here were obtained about two years ago. In the meantime we have completed several endocrine studies in this connection (4–9) the results of which afford a clear picture of the relation of water to creatine and creatinine metabolism. In these studies it was shown that any hormone that caused a retention of salt or water in the body at the same time caused an increased excretion of creatine. That creatinine is the mother substance of this creatine will be shown in this paper.

The following points were studied. a) The comparison of the effect of water and physiological saline upon creatine and creatinine excretion in the rat and man. b) the effect of injected saline, with and without creatinine, upon creatine excretion in the normal and castrate rat. c) The effect of alkalosis, with and without creatinine, upon

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creatine-creatinine excretion. *d*) The effect of injected saline on tissue creatine in the rat.

EXPERIMENTAL

Young adult rats weighing between 150 and 200 gm. were used. The feeding, caging and experimental technic have been described previously (6, 7, 9, 13, 17). The urines were collected under toluene for 2-day periods from both the rat and man. Total creatinine was determined by autoclaving the sample with HCl, followed, after neutralization, by the addition of 10% NaOH. Preformed creatinine was determined in the usual way by adding alkaline picrate to the urine sample. The difference between these values was expressed creatine, as creatinine, in the tables. Muscle creatine was determined by using the method of Rose, Helmer and Chanutin (10). The Fisher electrophotometer was used in all cases, for color matching. In all cases in which the ceatinine enzyme of Miller and Dubos (2) was used, the color of the Jaffe reaction disappeared showing that these large increases in color were due to creatinine alone.[2] The gonads were removed from male rats under ether anesthesia.

The control creatine and creatinine output of a given group would be determined for a 2-day period. It was then assumed that, had no changes in technic been instituted, this same amount of creatine and creatinine would have been excreted during each succeeding 2-day period. On the morning of the 3rd day, saline, water, creatinine, etc. were injected. The creatine and creatinine excretion was then determined for given periods of time. The following example illustrates the method of the calculation.

The excretion of creatine was 0 and of creatinine 15 mg. during the 2-day control period. This would be equivalent to 0 mg. of creatine and 150 mg. creatinine in the following 10 two-day periods. One cc. of water was injected on the third day and 20 days later the total excretion of creatine was 70 mg., and of creatinine 225 mg. Hence the 'extra' excretion of creatine was 70 mg. and of creatinine 75 mg. which was assumed to be due to the injection of the 1 cc. of water, etc.

RESULTS AND DISCUSSION

Effect of water and saline injection on creatinine-creatine excretion in rats. The injection of 1 cc. of water into the rat caused an extra excretion of 75 mg. of creatine within 22 days after injection (table 1). In previous studies the average amount of extra creatine excreted under these conditions was 35 to 40 mg. The injection of 2 cc. of water caused an extra creatine excretion of 113 mg. and 37 mg. of creatinine excretion. After the injection of 6 cc. of water the excretion values were no greater than after the injection of smaller amounts since most of this extra water was rapidly excreted. When the study was repeated using the same animals only 10 mg. of extra creatine was excreted with no change in creatinine excretion. The same phenomenon had occurred in both the rat and man in a previous study on creatinine injection or ingestion (1). The experimental period in this second study was only 4 days since there was little or no effect on creatine-creatinine excretion. At this time 1 to 4 cc. of physiological saline was injected into these animals. An average increase of 65 mg. of creatine excretion was accompanied by an average retention of 40 mg. of creatinine. These results were exactly the same as those reported above and it is possible that there was slightly more than a quantitative transformation of creatinine into creatine. A possible explanation of the effect of water on creatine excretion is furnished by the results of

[2] The mechanism of the reaction between creatinine and alkaline picrate has recently been studied by Dr. William M. McCord of this laboratory. Bolliger's (11) *Compound IV* ($creatinine_2$—$picric\ acid_2$—$NaOH_7$) was made up according to his method and used as an absolute standard. From 10 to 20 cc. of saturated picric acid and 1.3 to 2 cc. of 10% NaOH gave the maximum color development with a creatinine solution in 3 to 4 minutes, with little fading occuring during the first two hours. The electrophotometer was used. It was concluded that the Jaffe reaction under these conditions was a pseudo-monomolecular reaction.

Hevesy and Hofer (12) who showed that a molecule of heavy water remained in the body for 2 weeks.

From these results it is possible to say that water causes a transformation of body creatinine into creatine with little effect on body creatinine excretion, and that saline

TABLE 1. EFFECT OF PARENTERAL INJECTION OF WATER AND SALINE UPON CREATINE-CREATININE EXCRETION IN RATS.

Rats	Injection Substance		Average extra excretion of		Average retention of creatinine
	Water	Saline	Creatine, (as creatinine)	Creatinine	
No.	*cc.*	*cc.*	*mg.*	*mg.*	*mg.*
		experimental period 22 days			
4	1		75		
4	2		113	37	
2	6		112	10	
2	6		87	11	
		Av.	97	14	
3		1	70		70
3		2	95		22
3		3	50		26
3		4	46		35
		Av.	65		39
		experimental period 4 days			
3	1		5		
3	2		13		
3	3		12		
3	4		10		
		Av.	10		
		experimental period 20 days			
3		1	72		74
3		2	90		22
3		3	55		27
3		4	50		37
		Av.	67		40
Normal rats		*experimental period 8 days*			
10	16		147 } 169		5 } 46
10	16		22 }		41 }
Castrate rats		*experimental period 8 days*			
10	16		101 } 121		
10	16		20 }		

causes a retention of body creatinine and transforms it into creatine. Since more water would probably be retained in the presence of extra salt than when water alone was injected it is possible that this extra retention of water would account for the increased creatine excretion accompanied by an increased creatinine retention. There was no change in urine volume following injections 1 and 2 cc. of water. When 6 cc. was injected a slight increase in urine volume occurred. Injection of the same volume of water with salt was followed by a slight reduction in urine volume.

When massive doses of salt (144 mg.) were injected into normal rats in 8 cc. of water daily for 2 days there was an increased excretion of 147 mg. of creatine accompanied by a retention of 47 mg. of creatinine. In the same rats after castration the extra

creatine excretion was 101 mg. without any effect on creatinine excretion. Most of the extra creatine was excreted within 8 days after saline injection. A second study on the effect of water and salt for the next 8 days showed only small increases in extra creatine excretion, 22 and 20 mg., respectively. It is, therefore, possible that the retention of water under the influence of saline was responsible for the creatinine to creatine transformations noted in these saline studies. It is also evident that body creatinine was transformed into creatine in the presence of the gonads, and that in their absence saline caused only a stimulation of creatine excretion without changing the creatinine excretion to any appreciable degree.

TABLE 2. EFFECT OF WATER AND SALT INGESTION UPON URINE VOLUME AND CREATINE-CREATININE EXCRETION IN MAN

Subject	Control period (16 days)				Experimental period (28 days)				
	Total water intake	Total urine volume	Total excretion of extra		Total water intake	Total salt intake	Total urine volume	Total excretion of extra	
			Creatine (as creatinine)	Creatinine				Creatine (as creatinine)	Creatinine
	liter	*liter*	*gm.*	*gm.*	*liter*	*gm.*	*liter*	*gm.*	*gm.*
P.P.	36.5	40.6	3.9	2.3	48.7	10	50.5	5.7	3.8
H.H.B.	24.2	24.6	3.2	10.2	46.4	20	44.8	11.2	5.3

Effect of water and saline ingestion on creatine-creatinine excretion in man. The data listed in table 2 show the relation between water exchange and creatine-creatinine excretion in man. *Subject PP* ingested 5 liters of water during the third 2-day period. This was 2 liters above the usual water intake during this period. Four liters more of urine were excreted than the water ingested during the next 16 days. The increase in extra creatine and creatinine excretion was 3.9 and 2.3 gm. respectively. In the next period this subject ingested 10 gm. of NaCl in 8 liters of water during the third 2-day period. Slightly more urine was excreted in the next 28 days as compared to the water intake during this period. The increase in extra creatine and creatinine excretion was 5.7 and 3.8 gm. respectively. This was about double their excretion with water alone. *Subject HHB* ingested 5 liters of water during the third 2-day period. He was in water balance during the next 16 days. But the extra creatine and creatinine excretion was 3.2 and 10.2 gm. respectively during this time. During the third 2-day period in the second study he ingested 20 gm. of NaCl in 6 liters of water. During the next 28 days he ingested 46.3 liters of water and excreted 44.8 liters of urine. A retention of 1.5 liters of water occurred. The extra creatine excretion was 11.2 gm. and creatinine excretion, 5.3 gm. respectively, as compared to their excretion when water alone was ingested. Even though both subjects were essentially in water balance the sudden increase in water and salt intake had a pronounced effect upon creatine and creatinine excretion.

Effect of injection of saline, with and without creatinine, upon creatine excretion in normal and castrate rats. The results listed in table 3a show an average quantitative summation in creatine excretion when saline and creatinine were injected together as compared to their separate injections (83.3 mg. as compared to 42.4+40.9 mg.). This did not occur in the same male rats after castration (46.2 vs. 50.6 mg.). The creatinuria here was due to the injected saline alone (table 3b). These findings confirm those of Beard and Jacob (6, 7) and again show that the transformation of exogenous creatinine

TABLE 3. EFFECT OF PARENTERAL INJECTION OF PHYSIOLOGICAL SALINE AND CREATININE UPON (*a*) CREATININE-CREATINE AND (*b*) CREATINE-CREATININE EXCRETION IN RATS. EXPERIMENTAL PERIOD, 20 DAYS

Rats	Saline Injected	Creatinine			Extra Creatine (as Creatinine) Excreted	
		Injected	Extra Excreted	Retained		
no.	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	
			(a) Simultaneous and separate administration, normal rats			
2	2	—	—	—	32.0	61
2	1	20	—	20	(tr)[1] 29.6	
3	2	20	40	—	(s)[2] 76.0	
2	4	—	23	—	56.0	79
2	1	40	25	15	(tr) 23.0	
3	4	40	51	—	(s) 75.0	
2	6	—	—	—	53.0	81
2	1	60	29	31	(tr) 28.8	
3	6	60	35	25	(s) 67.0	
2	8	—	25	—	53.6	83
2	1	80	40	40	(tr) 30.0	
3	8	80	7	73	(s) 67.0	
2	10	—	46	—	39.0	79
2	1	100	55	45	(tr) 40.0	
3	10	100	117	—	(s) 101.0	
2	2	—	—	—	32.0	97
2	2	150	110	40	(tr) 65.0	
3	10	150	120	30	(s) 92.0	
2	2	—	—	—	32.0	102
2	2	200	91	109	(tr) 70.0	
3	10	200	167	33	(s) 99.0	
Av. creatine excretion, salt and creatinine together, 82.4 mg.; separately, 83.3 mg.						
			(b) Castrate rats			
2	2		None		33	
	2	20	None	20	43	
2	4		None		51	
	4	40	9	31	44	
2	6		None		39	
	6	60	18	42	41	
2	8		14		59	
	8	80	40	40	69	
2	10		35		49	
	10	100	76	24	56	
Av. creatine excretion, salt and creatinine together, 50.6 mg.; with salt alone, 46.2 mg.						

[1] tr = transformation of creatinine to creatine.
[2] s = stimulation of creatine excretion.

to creatine takes place only in the presence of the gonads in rats. In the castrates saline again stimulated creatine excretion.

The data listed in table 3 are also interesting from another point of view. When amounts of creatinine from 20 to 200 mg. were dissolved in 1 or 2 cc. of saline and in-

jected the average creatinine retention in 8 studies was 46.6 mg. This was accompanied by an average increased creatine excretion of 44.2 mg. We believe that this may be taken as an indication of a quantitative transformation of the retained creatinine into creatine. On the other hand if these same amounts of creatinine were dissolved in 4 to 10 cc. of saline and injected the average creatinine retention was only 14.6 mg. accompanied by an average increased creatine excretion of 85 mg. In these cases the large amount of saline injected caused a stimulation of creatine excretion.

The increase in muscle creatine after 3 days of water starvation was directly proportional to the increase in muscle solids (table 4).

TABLE 4. EFFECT OF WATER STARVATION UPON TISSUE CREATINE IN RATS

No. of animals	Muscle Total Solids		Creatine Content of Muscle	Liver	Kidney	Heart	Remarks
	%		%	%	%	%	
30	26.2	max.	0.44	0.08	0.16	0.26	Normal controls
	23.8	min.	0.37	0.06	0.08	0.19	
	25.1	av.	0.42	0.07	0.12	0.21	
4	29.4	max.	0.70	0.15	0.35	1.10	Three days of water starvation
	28.1	min.	0.43	0.11	0.20	0.75	
	28.9	av.	0.59	0.12	0.33	0.87	
4	27.0	max.	0.41	0.08	0.14	0.41	Three days of water starvation +2 days of H_2O
	25.0	min.	0.38	0.04	0.09	0.28	
	25.5	av.	0.39	0.06	0.11	0.32	

Effect of injection of physiological saline upon tissue creatine. It was of interest to determine if the large doses of water or saline were simply 'washing out' the tissue creatine into the urine. That this did not occur is shown by the results listed in table 5. No change in muscle creatine occurred until a massive dose (1.2 gm.) of saline was injected during the course of 3 days. The average increase in muscle creatine here was 29% above normal. This increase of saline would not, however, arise during the course of normal metabolism in the rat. No changes in liver or kidney creatine occurred but the increase in heart creatine ranged from 20 to 70% above normal. This finding might indicate that some of the creatinuria observed in cases of cardiac edema might be due to the edema *per se*.

The fact that the injection of saline does not cause an increase in muscle creatine is of importance for the interpretation of the results secured by Beard and Pizzolato (13) on the concentration of muscle creatine upon injecting the amino acids. These increases were due to the amino acids and not to the 1 cc. of saline that they were dissolved in.

Effect of sodium bicarbonate and disodium phosphate feeding upon creatine-creatinine excretion in the rat. The effect of feeding 1 or 2% of our stock diet as disodium phosphate or sodium bicarbonate on creatine-creatinine excretion is shown in figure 1. Large and significant increases in creatine excretion occurred especially after feeding the 2% diets. At the end of the 8th experimental day each animal received 40 mg. of creatinine mixed with about 5 gm. of his stock diet. This was quickly consumed, after which the stock diet was replaced in the cage. On the phosphate diet with creatinine 82% of the ingested creatinine was retained and 37% of this was transformed into creatine and excreted. On the bicarbonate diet 75% of the injected creatinine was retained and 77% of it was transformed into creatine and excreted. These urines con-

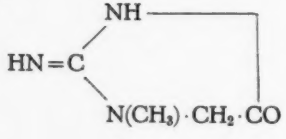
tained no sugar or acetone bodies. It is possible that alkalosis was responsible for these changes.

TABLE 5. EFFECT OF SALINE INJECTION UPON CONCENTRATION OF TISSUE CREATINE IN THE RAT

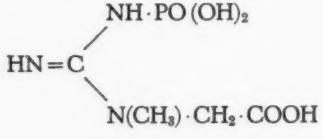
Saline Injected	Salt Content	No. of Animals	Experiment		Creatine Content of			
					Muscle	Liver	Kidney	Heart
cc.	mg.		days		%	%	%	%
Controls		30	2	max.	0.44	0.08	0.16	0.26
				min.	0.37	0.06	0.08	0.19
				av.	0.42	0.07	0.12	0.21
2	18	3	2	max.	0.41	0.06	0.10	0.28
				min.	0.39	0.04	0.09	0.25
				av.	0.41	0.05	0.10	0.27
4	36	4	2	max.	0.48	0.06	0.11	0.38
				min.	0.42	0.05	0.09	0.30
				av.	0.45	0.05	0.10	0.32
6	54	4	2	max.	0.47	0.06	0.11	0.33
				min.	0.43	0.04	0.09	0.21
				av.	0.44	0.05	0.10	0.27
8	72	4	2	max.	0.44	0.08	0.11	0.29
				min.	0.40	0.06	0.09	0.23
				av.	0.41	0.07	0.10	0.25
10	90	4	2	max.	0.43	0.08	0.12	0.38
				min.	0.39	0.06	0.09	0.30
				av.	0.41	0.07	0.11	0.33
16	144	10	2	max.	0.48	0.09	0.14	0.36
				min.	0.38	0.04	0.09	0.28
				av.	0.42	0.06	0.12	0.32
24*	1200	5	4	max.	0.58	0.09	0.15	0.43
				min.	0.50	0.06	0.09	0.30
				av.	0.54	0.08	0.11	0.35

* 100 mg. in 2 cc. water, t.i.d. = 300 mg. daily for 4 days.

There may be an important relation between creatinine and phosphocreatine in metabolism which has been overlooked in the past. If one considers the structures of these compounds, the free amino group of the guanidine moiety is attached to an acid group,



Creatinine



Phosphocreatine

Both Wang (14) and Lippmann (15) have also referred to this chemical relationship. Wang believes that creatinine arises from the total creatine of the tissues rather than from free creatine. Lippmann believes that phosphocreatine may be the mother substance of the urinary creatinine: "With the tension present in the P-N linkage a change to the more stable and similar C-N linkage, offered by the presence of carboxyl in the molecule, seems suggested."

Since the hydrolysis of phosphocreatine during muscular contraction does not necessarily cause an increased excretion of either creatine or creatinine Lippmann's theory is untenable. Chakelis (16) reported a retention of creatinine in muscular exercise. Beard (17) observed that creatinine ingestion by medical students would serve to increase their energy output above the control value, while creatine ingestion did not do so and it was suggested that the retained creatinine observed by Chakelis might have been transformed into creatine, which as phosphocreatine served to increase the energy output. Our results may serve as a basis to picture the process as follows:

Tissue Creatinine + Phosphate (H_2O) → Phosphocreatine → Tissue Creatine
↓ ↓
Urine Creatinine Urine Creatine

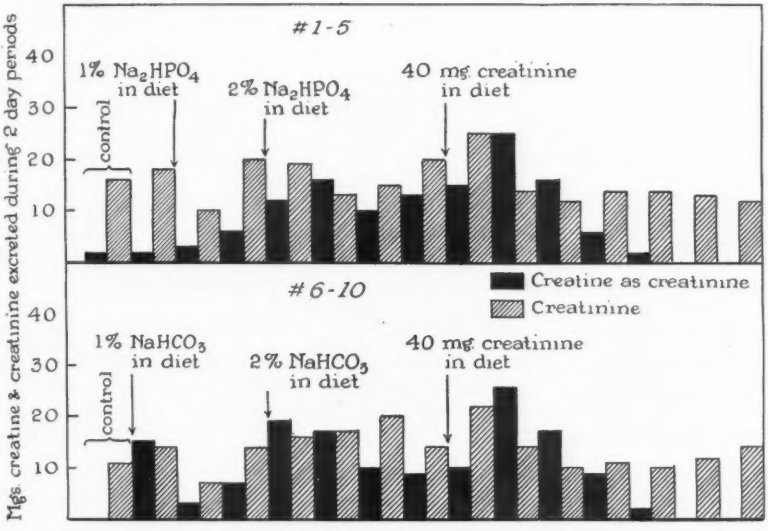


Fig. 1. Effect of feeding disodium phosphate and sodium bicarbonate, with and without creatinine, upon creatine-creatinine excretion.

Creatine formed from the amino acids is functional creatine. It combines with phosphorous to form phosphocreatine. That formed from creatinine may likewise be stored in the muscles and utilized in the same way. Abdon (18) recently reported that the phosphocreatine content of the blood was increased after the administration of either creatine or creatinine. In any case the concentration of phosphate in the tissues determines the magnitude of creatine formation and retention. On the other hand creatine formed from water or saline is either non-functional or is excreted faster than it can be stored. The formation of both creatinine and creatine in the body is a steady-state reaction under the usual metabolic conditions and any excess of these substances not stored is then excreted into the urine.

Hawk and Fowler (19) observed a total excretion of extra creatine of 1.45 gm. in their subject who drank 3 liters of water daily with his meals for 3 days. Morgulis and Osherhoff (20) reported marked increases in Na, Cl and Ca in dystrophic rabbit muscle. Fenn and Goettsch (21) found a considerable increase in extracellular water in

muscle tissue during nutritional muscular dystrophy. Morgulis and Spencer (22) showed that, at the 'critical point' in rabbits suffering from nutritional muscular dystrophy, there was a marked diuresis and an increase in creatine and phosphate excretion. It is, therefore, possible that some of the creatine excreted in the urine of the myopathic patient might have its origin in part from the increased water content of the tissues as well as from creatine from the muscle tissue itself. After infusion of cyanol or euphyllin into rabbits Descombes (23) reported that the percentage of creatinine retention was usually proportional to the increase in diuresis.

Data from studies on diabetes insipidus are conflicting. D'Antona (24) stated that creatine-creatinine metabolism in this disease was essentially normal. Hanssen (25) reported that the administration of posterior pituitary powder reduced the diuresis and increased creatinine excretion. Pákozdy (26) stated that the creatinine excretion in diabetes insipidus varied from 1.1 to 2.7 gm. per day and that treatment with posterior pituitary reduced the diuresis and excretion of creatinine.

Beard et al. (4–9) have shown that the injection of the following hormones causes a creatinuria in normal rats followed, in some cases, by creatinine retention: theelin testosterone propionate, anterior pituitary growth, sex and thyrotropic hormones, follutein, and progesterone. This was especially true after the injection of the posterior pituitary hormones (9) and the cortical hormone of the adrenal cortex (8). The salt and water-retaining properties of theelin, testosterone propionate and progesterone, have been reported by Krohn and Zuckerman (27) and Thorn and Harrop (28). It is therefore, fairly certain that our results can be explained on the basis of water and salt retention after injection of the different hormones. This would favor the retention of creatinine and its transformation into creatine at the same time.

The results of our studies may also explain the so-called constancy of creatinine excretion. The creatinine to creatine transformation is a steady-state reaction, the excess creatinine not transformed into creatine being excreted as such into the urine. Its excretion would depend, not upon body weight, but upon a uniform protein and water intake, resulting in a nitrogen and water balance. Any factor, condition, or set of conditions that affects the usual rate of metabolism will, at the same time, affect the rate of creatine and creatinine metabolism.

SUMMARY AND CONCLUSIONS

The results reported in this paper are based upon a study of the following points: *a*) A comparison of the effect of injection and ingestion of water and physiological saline upon creatine-creatinine excretion in the rat and man. *b*) The effect of injected saline upon tissue creatine in the rat. *c*) The effect of saline with and without creatinine upon creatine and creatinine excretion. *d*) The effect of feeding 1 or 2 % of the stock diet as sodium bicarbonate and disodium phosphate upon creatine-creatinine excretion.

Injection of water or of saline into the rat resulted in the same degree of extra creatine excretion.

Water caused a transformation of body creatinine into creatine without body creatinine retention while salt caused a retention of body creatinine and its transformation into creatine.

Massive doses of saline caused a slightly larger stimulation of creatine excretion in normal as compared to castrate rats. Some body creatinine retention occurred in the normal rats but not in the castrates.

Ingestion of saline by man greatly increased the amount of creatine and creatinine excreted over that observed from the ingestion of the same amount of water alone with one exception.

Simultaneous injection of saline and exogenous creatinine into rats doubled the average creatine excretion usually found following the injection of the same amounts of saline and creatinine separately. The creatinuria in these same rats after castration under these conditions was due to a stimulating effect of water or salt upon creatine excretion. The presence of the gonads was necessary for the transformation of exogenous creatinine into creatine. The retained exogenous creatinine which was injected in 1 or 2 cc. of saline was, on the average, quantitatively transformed into creatine; these same doses of creatinine injected in 4 to 10 cc. of saline stimulated creatine excretion. The injection of water or saline did not increase the creatine content of tissue, except in the heart in a few cases. Increases in muscle creatine were directly proportional to the total solid content of the muscles in 3-day water-starved rats. Feeding of 1 or 2% of the stock diet as sodium bicarbonate or disodium phosphate greatly increased the creatinine to creatine transformation and excretion in the rat. The results of the present study, as well as the results of a later study on the effect of creatinine ingestion upon energy output in medical students, suggested the theory that in the presence of water and phosphate creatinine is changed into phosphocreatine, which then serves to supply energy for muscle contraction processes.

REFERENCES

1. Jacob, E. J., and H. H. Beard: *Arch. Neurol. and Psychiat.* 42: 67. 1939.
2. Miller, B. F., and R. Dubos: *J. Biol. Chem.* 121: 457. 1937.
3. Brazda, F. G., J. K. Espenan and H. H. Beard: *Pacific Coast Med.*, 7: 38. 1940.
4. Pizzolato, P., and H.H. Beard: *Enocrinology* 24: 358. 1939.
5. Koven, A. L., and H. H. Beard *Endocrinology* 25: 221. 1939.
6. Beard, H. H., and E. J. Jacob: *Endocrinology* 26: 1064. 1940.
7. Beard, H. H., and E. J. Jacob: *Endocrinology* 26: 1069. 1940.
8. Koven, A. L., P. Pizzolato and H. H. Beard: *Endocrinology* 27: 95. 1940.
9. Beard, H. H., and P. Pizzolato: *Endocrinology* 27: 908. 1940.
10. Rose, W. C., O. M. Helmer and A. Chanutin: *J. Biol. Chem.* 75: 543. 1927.
11. Bollinger, A.: *Proc. Soc. N. S. Wales* 70: 357. 1937.
12. Hevesy, G., and E. Hofer: *Klin. Wochnschr.* 13: 1524. 1934.
13. Beard, H. H., and P. Pizzolato: *J. Biochem.* 28: 421. 1937.
14. Wang, E.: Clinical and Experimental Investigations on the Creatine Metabolism, Mercatros Tryckeri, Helsingfors, p. 278. 1939.
15. Lippmann, F.: Advances in Enzymology, 1: 118. 1941. Interscience Publishing Co., New York.
16. Chakelis, A.: *Am. J. Physiol.* 132: 578. 1941.
17. Beard, H. H.: *Modern Med.* In press.
18. Abdon, N-O, Kungl.: Fisiografiska Sallsk. I. Lund. Forhandlingar, 5: 1. 1935.
19. Hawk, P. B., and C. C. Fowler: *J. Exp. Med.* 12: 388. 1910.
20. Morgulis, S., and W. Osherhoff: *J. Biol. Chem.* 124: 767. 1938.
21. Fenn, W. O., and M. Goettsch: *J. Biol. Chem.* 120: 41. 1937.
22. Morgulis, S., and H. C. Spencer: *J. Nutrition* 12: 191. 1936.
23. Descombes, E.: *Biochem. Ztschr.* 246: 59. 1932.
24. D'Antona, L.: Arch. de Inst. biochem. Ital. 3: 187. 1931.
25. Hanssen, P.: *Hospitalstid.* 78: 1306. 1935.
26. Pákozdy, K.: *Ztschr. f. klin. Med.* 112: 442. 1929–30.
27. Krohn, P. L., and S. Zuckerman: *J. Physiol.* 88: 369. 1937.
28. Thorn, G. W., and G. A. Harrop: *Science* 86: 40. 1937.

Addendum. We have recently completed a study of the equilibrium of creatine and creatinine incubated, with and without the addition of various tissues at 37°C., for various lengths of time. Creatinine was both oxidized and hydrated to creatine while creatine was oxidized but was not transformed into creatinine in the presence of any tissue. The presence of 3 enzymes in these various rat tissues was shown and named *creatinine oxidase* and *hydrase* and *creatine oxidase*. Creatine anhydrase does not occur in these tissues.

LESIONS OF THE BLADDER AND OF THE PROSTATE OF MICE RECEIVING PELLETS OF CRYSTALLINE ESTRONE

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THE FOLLOWING OBSERVATIONS WERE MADE in two groups of animals used for the study of the influence of crystalline estrone on the course of chemically-induced cutaneous and subcutaneous tumors. The observation of lesions in the bladder mucosa, the frequency of which appeared great enough to warrant discussion, will serve as the basis for this report.

Histological studies were made on 25 male and female mice from a total of 60 animals treated with estrone. 20 of these were C_3H mice obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and 5 were albino stock mice. Into all animals at the age of 7 weeks, a small pellet of compressed crystalline theelin[3] was implanted subcutaneously through a small opening in the skin of the lower abdomen at the outset of the experiment. A second pellet was implanted at the same site in the C_3H mice 7 weeks later. The total weight of the pellets ranged from 4.1 to 6.7 mg. (av. 5.1 mg.) in the C_3H and from 0.75 mg. to 2.4 mg. (av. 1.5 mg.) in the stock animals. The average duration of stimulation with estrone had been 3.8 months in the former and 5.1 months in the latter group of animals.

Eleven C_3H mice received in addition, 0.5 mg. of methyl-cholanthrene dissolved in 0.25 cc. of sesame oil. This was injected into the subcutaneous tissue of one groin. To the 5 albino mice from 3 to 8 drops of 1, 2: benzpyrene (0.3%) dissolved in benzol were administered to the skin of the back with a medicine dropper 3 times weekly, until papillomata appeared. Of the 11 animals inoculated with methylcholanthrene, 7 showed tumors of varying sizes at autopsy. All 5 benzpyrene animals developed papillomata.

The tissues were fixed in Bouin's fluid, dehydrated in alcohol and embedded in paraffin. Serial sections were made of portions of the organs, including the prostate, seminal vesicles and bladder. The sections were stained with hematoxylin and eosin.

The effects of estrogenic substances on the primary and secondary sex organs of normal and castrated male and female mice have been extensively studied and described (1, 2, 3). The characteristic lesions that develop in treated normal male mice consist of epithelial changes leading to and terminating in a progressive squamous metaplasia of the prostate, the coagulating gland, the urethra and periurethral glands. There has also been noted an increase in the fibromuscular tissues of these organs. In the female animal a marked proliferation of the vaginal and cervical epithelium with extensive keratinization and, in addition, squamous metaplasia of the endometrium

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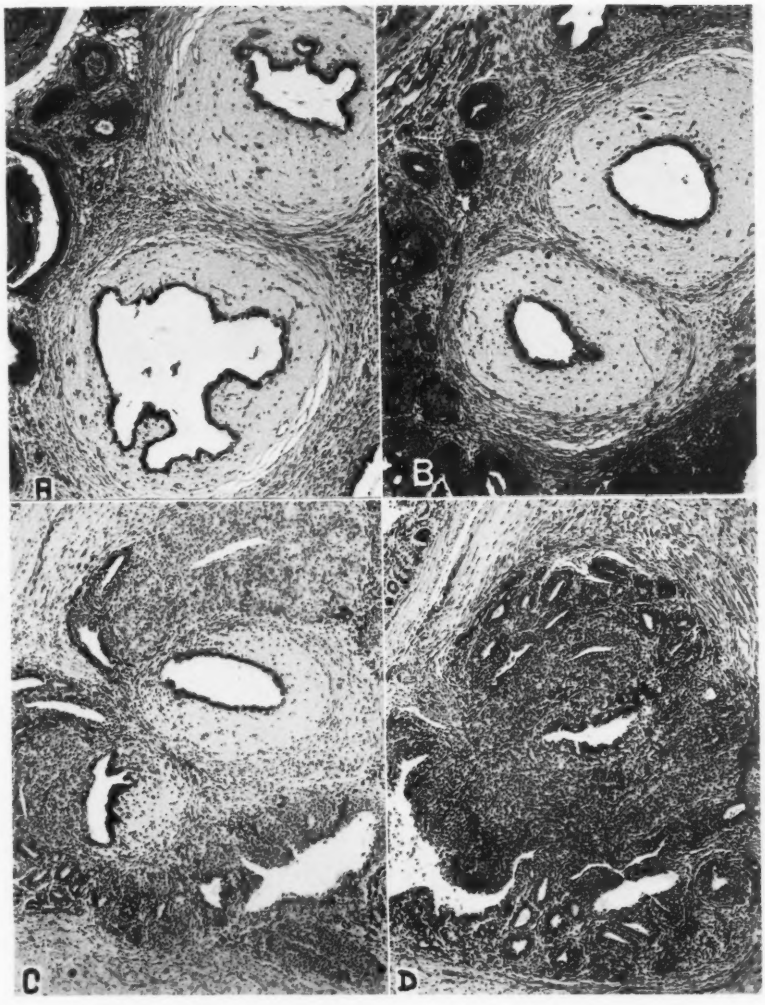

Fig. 1, A–D. SERIAL SECTIONS OF PROSTATE AND EJACULATORY DUCTS. Squamous metaplasia of prostatic glands destroying ejaculatory ducts. For details see description in text H&E stain ×58.

have been described. This has often been followed by uterine infection (pyometrium). An increase in the stromal connective tissue of the uterus has also been observed (3).

Identical lesions varying in degree have been observed in most of the animals of our series. In one animal the proliferation of the metaplastic squamous epithelium of the prostate had become so extensive that a more detailed description seems justified.

The extent of the lesion in this animal can best be described in conjunction with photographs of serial sections (fig. 1, A–D). Figure 1, A shows the ejaculatory ducts surrounded by loose connective tissue. The epithelium of the duct is slightly hyper-

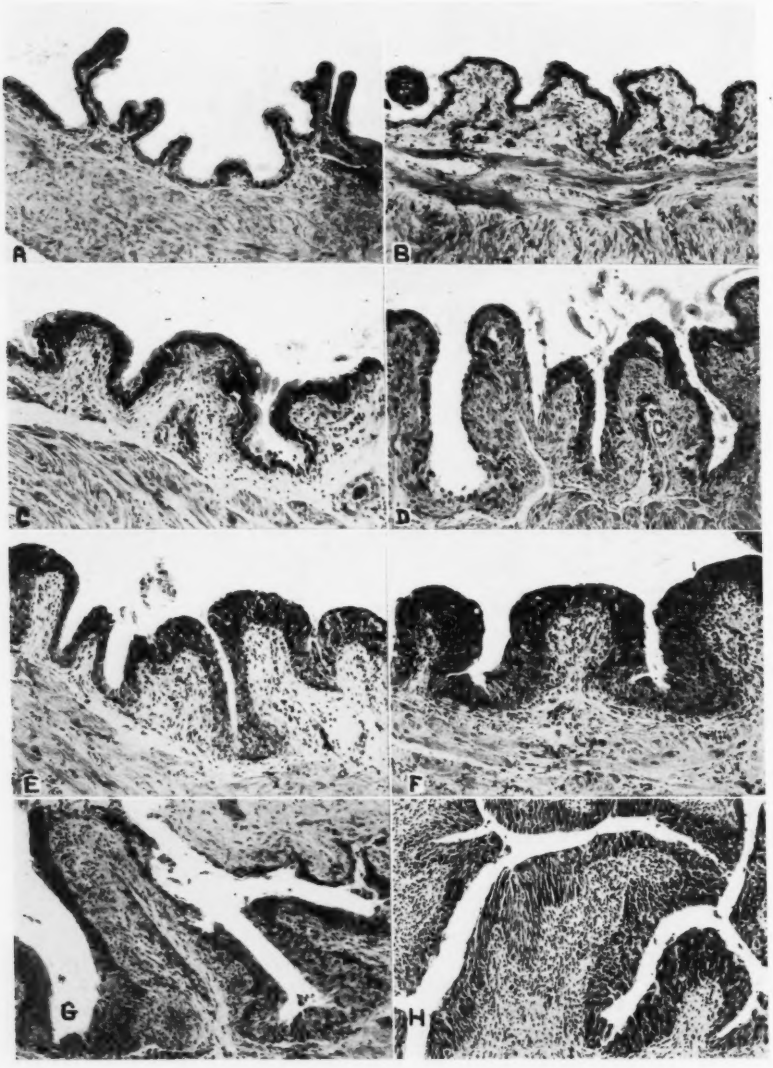

Fig. 2, A–H. Sections of urinary bladders. *A*: control animal; *B–H*: different degrees of squamous metaplasia of bladder epithelium. For details see description in text. H&E stain ×93.

plastic. The prostatic glands in the adjacent tissue exhibit the familiar squamous metaplasia with and without keratinization and dilatation of acini. There is slight inflammatory infiltration of the dense stroma. Figure 1, B shows more extensive masses of keratinized squamous epithelium with pearls encroaching upon the periductal tissue. The squamous cells grow outwardly from the prostatic ducts into the surrounding connective tissue. This, however, takes place in an expansive rather than in an in-

filtrative manner. There is beginning focal metaplasia of the epithelium of the opposite prostatic duct. In figure 1, C, a more extensive proliferation of squamous cells is seen; the cells have destroyed a portion of the lining of one ejaculatory duct and a few islands of squamous cells are seen in the loose periductal stroma. The other ejaculatory duct is now also encroached upon by similar cells. Figure 1, D shows the first duct to be completely replaced by masses of squamous cells and the second duct is also almost completely destroyed.

This series of photomicrographs clearly demonstrates the extent to which the metaplastic squamous cells may proliferate under the influence of estrone. Although they are neither anaplastic nor malignant in character they grow in an expanding tumor-like fashion, replacing adjacent structures. They are without true infiltrative characteristics.

In addition to these changes in the secondary sex organs, certain alterations in the epithelium of the urinary bladder were observed in 16 of the 25 animals.

The normal mouse bladder has a single or double layer of epithelial cells. The basal layer consists of flattened cells whereas the cells in the upper layer exhibit a more or less prominent cuboidal shape and often have a granular cytoplasm. There may be as many as 3 layers of epithelial cells in a contracted bladder and small papillary projections are found (fig. 2, A). The submucosa consists of a few strands of fibrillar connective tissue in which blood vessels are seen. The muscular wall consists of bundles of smooth muscle fibers. A few perivascular lymphocytes may occasionally be seen.

The changes in the urinary bladder produced in our animals occurred primarily in the epithelium. In some animals variable degrees of inflammation were seen. In these there was a predominantly polymorphonuclear exudate in the lumen and desquamation of epithelium. A few of the bladders showed remnants of normal epithelium and in its place a multi-layered squamous epithelium with varying degrees of differentiation. The character of the epithelium varied from animal to animal. What appeared to be 2 different stages in the development of the process were observed. In the early phase the mucosa was well preserved and intact. Distinct thickening of the epithelium was present in many places with areas of increased cellularity and of proliferation of basal cells. The superficial layer of epithelial cells was often desquamated and free cells were seen in the lumen. There were small foci of true squamous epithelium. Islands of normal transitional mucosa alternated with areas in which the above described abnormalities were noted. Little or no evidence of inflammation was seen in these tissues and an increase in thickness of the submucosa and of the muscularis was present in some. The papillary projections composed of mucosa and submucosa were much larger than those in the contracted bladder of normal animals.

It was this metamorphosis of the thin transitional type of epithelium into one with increased cellularity and density containing islands of stratified squamous epithelium which seemed to represent the earliest form of the process. As a rule these alterations were accompanied by slight or no inflammatory reaction. They were present in 8 of the 16 animals with bladder lesions (fig. 2, B–F).

The remaining 8 animals showed a more advanced degree of metaplasia. In most of these it was associated with intense inflammatory reaction and very considerable thickening and vascularity of the submucosa. Large areas of submucosal tissue were denuded of epithelium and clumps of bacteria were occasionally noted. The intact mucosa consisted of a multi-layered very cellular stratified epithelium in which mitotic figures were frequently observed. No abnormalities of nuclear division were noted. The nuclei of these cells varied a great deal in shape and size. Some were vesicular while others contained a dense chromatin network. The shape of the cells likewise

varied from place to place; most of them were elongated and spindle-shaped, arranged in palisade-like fashion in several layers. Intercellular bridges were not formed. Kerato-hyaline bodies were occasionally observed. The mucosa and submucosa was thrown into folds which were still larger than those in the preceding group of animals. This was obviously due to the marked thickening of the submucosa. The muscular wall was thickened and showed inflammatory reaction varying in degree and character (fig. 2, G–H).

Sixteen of the 25 mice whose tissues were studied microscopically exhibited lesions of the bladder. Of the remaining 9 animals, 4 showed an advanced degree of autolysis

TABLE 1. RESPONSE OF BLADDER EPITHELIUM IN MICE TO PROLONGED STIMULATION WITH ESTRONE

Animals			Av. wt. of estrone pellet	Duration of stimulation		No. of animals showing			
Strain	Sex	No.		Av.	Range	Early lesion	Advanced lesion	Negative response	Autolysis
			mg.	*mo.*					
C_3H	♂	10	5.6	4.3	3.0 6.2	3	3	3	1
C_3H	♀	10	4.6	3.3	2.0 4.3	1	5	2	2
Stock	♂	2	1.5	5.1	5.0 5.3	1			1
Stock	♀	3	1.6	5.1	5.0 5.3	3			
Total ♂+♀		25				8	8	5	4

precluding accurate interpretation, although in 2 of these epithelial and particularly sub-mucosal changes were suggestive and comparable to those of the second group of animals. Five animals showed no lesions in the bladder. Three were males and at autopsy exhibited an extremely dilated bladder containing a rounded mass of soft amorphous whitish yellow material. The epithelium of these bladders was largely desquamated and a few hemorrhages were seen. All 3 animals showed the effect of estrone in the prostate. The remaining 2 female mice failed to show any evidence of alteration and the vaginal epithelium exhibited only very slight evidence of estrogenic activity. It may be added that there were just as many animals with bladder lesions in the group injected with methylcholanthrene as there were in the uninjected group. The findings are briefly summarized in table 1.

DISCUSSION

Lacassagne (4) described a progressive proliferation, thickening and budding of the mucosa in the bladders of a few mice injected with estrone. These changes were thought to be a direct local effect of accumulated estrone in the stagnating urine. According to Lacassagne this proliferative reaction was similar in character to that found in other organs, such as breasts, uterine glands, and prostate but was not brought about in the same manner, that is, by way of the blood stream. Raynaud (5) studying the effects of injections of estradiol on cats noted distinct thickening and squamous metaplasia with early keratinization of the epithelium of the bladder and marked thickening of the muscular wall. There was no dilatation of the bladder and no stagnation of urine. The lesions were seen after 7 injections of a total of 2 mg. of estradiol. Raynaud believed that the effect on the bladder was as specific as that on other organs. Murray and Steinkamm (6) studying the bladders of 86 rats found no evidence of

stimulative action by estrone on the bladder epithelium, but noted a marked increase in size and thickness of the musculature of the bladder in rats receiving estrone. A striking difference in the size existed among the normal, the castrated and the estrone-treated animals. The hyperplasia of the epithelium in some of their animals was thought to be due to the presence in the bladder of enormous numbers of a parasite, *Trichosoma crassicauda*. This parasite was found in 65% of the animals. A specific action of estrogenic substances on the bladder epithelium of rats did not exist according to these authors. No mention was made of epithelial changes in the bladder of mice by Burrows (1, 2). He described extreme dilatation of the bladder in many animals of both sexes. Since no direct obstruction to urinary outflow through the urethra was demonstrable, a disturbance in the nervous regulation of the bladder function was suggested as the cause of the dilatation.

The data presented in this report indicate that the bladder epithelium and musculature can be stimulated and influenced by the long-continued action of estrone. The animals showed metaplastic changes of the epithelium quite similar to those observed in the prostate, urethra, and in other organs. There also occurred an apparent increase in the mass or in the size of muscle fibers of the wall. The latter could perhaps also be explained as a compensatory hypertrophy due to the relative obstruction of the urethra produced by metaplastic and hyperplastic changes of the epithelium and increased density of periurethral stroma. The data of Murray and Steinkamm (6) on the other hand showed such marked differences in the size of the bladders of normal, castrated and estrone-treated rats that one is inclined to accept a direct stimulative effect of estrogenic substances upon the bladder musculature as well. The observations of Raynaud would likewise favor such an interpretation. Although no direct proof of such action can be furnished by our experiments they strongly suggest that such a relationship exists.

The described epithelial changes of the mucosa are considered to be a direct effect of estrone on these cells. There is no reason to believe that these changes were due to a local action of estrone or excretion products in the urine, a view expressed by Lacassagne (4). No such local action is supposed to bring about the changes in the urethra which is so consistently altered in estrinized animals. Moreover, there is no reason to exclude the urinary bladder from the estrogen-responsive organs. The effect of estrogenic substances is manifested, according to Burrows (2), primarily in the organs directly concerned with reproduction, but may not be confined to them. Structures which have their embryologic origin in the same system may also respond. This may reasonably be said to be the case of the urethra and, on the basis of our studies, of the bladder as well. There is, furthermore, no evidence that the metaplastic changes in urethra or bladder are a result of inflammatory processes. It has been pointed out above that in the early stage of the process focal areas of hyperplasia and metaplasia can be demonstrated in association with gradual thickening of the submucosa without inflammation or residua of it. In the later stages, however, a presumably ascending urinary infection leads to marked inflammatory changes with desquamation of epithelium and ulceration. Clinical observations clearly demonstrate the close relationship between squamous metaplasia and luxuriant growth of bacteria in the organs affected. This is well known from the frequent occurrence of infection in organs with metaplastic changes in the case of animal and human vitamin A-deficiency. The frequent development of pyometrium is a manifestation of the same principle in estrinized animals. The metaplastic squamous cells form an ideal medium for the growth and spread of bacteria as has been pointed out by Blackfan and Wolbach (7). The cystitis is therefore considered to be a consequence rather than a precursor of the metaplasia

in our animals. The bladder epithelium is the least responsive structure to the action of estrone since the epithelial changes are usually much more marked in the urethra and in the periurethral glands. The advanced degree of squamous metaplasia of the bladders showing severe inflammation may be due partially to the rapid proliferation of cells in an attempt to cover the surface defects. Assuming that all of the epithelium is under the influence of estrone, a type of repair consisting exclusively of squamous epithelial cells may be expected. In fact no normal epithelium has been observed in any of the animals with severe lesions. No attempt has been made in this study to determine whether the metaplasia of the bladder is in part an extension of metaplastic epithelium of the urethra into the bladder. It appears from the sections of tissues that in a few animals early lesions are localized at the neck of the bladder. Other regions in the bladder at a distance from the urethral orifice may however react independently and apparently simultaneously. It is interesting to note that the albino mice have shown the early type of lesion only, in spite of the fact that they were under the influence of estrone for a longer period of time than the C_3H mice. However, the total dose of estrone administered to these animals was considerably smaller and this may perhaps account for the discrepancy in the final result at the time of death. No such relationship could be established for the C_3H group of animals showing early lesions of the bladder. Unfortunately no determinations of the total dose of estrone absorbed by the animals have been carried out, although in most of the animals remains of the pellets were recovered at autopsy.

SUMMARY

The effect on the reproductive system and the urinary bladder of crystalline estrone implanted in form of pellets into the subcutaneous tissue has been studied histologically in 20 C_3H and 5 albino stock mice of both sexes.

The animals were under the influence of estrone from 2 to 6.2 months, the average being 3.5 and 4.1 months respectively. The lesions of the reproductive system were identical with those described in the literature. The extraordinary extent to which the metaplastic epithelium of the prostate may proliferate under long continued stimulation has been illustrated. Hyperplastic and metaplastic lesions of the epithelium of the urinary bladder consisting of squamous metaplasia of varying degrees have been described and discussed. The lesions are identical with but not as conspicuous as those of other organs following the administration of estrone. They are interpreted as being due to the direct influence of the female sex hormone.

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REFERENCES

1. Burrows, H.: *Am. J. Cancer* 23: 480. 1935.
2. Burrows, H.: *J. Path. and Bact.* 41: 423. 1935.
3. Loeb, L., V. Suntzeff and E. L. Burns: *Am. J. Cancer* 34: 413. 1938.
4. Lacassagne, A.: *Compt. rend. Soc. de biol.* 120: 833. 1935.
5. Raynaud, A. *Compt. rend. Soc. de biol.* 126: 215. 1937.
6. Murray, E. M., and E. Steinkamm: *Arch. f. Gynaek.* 164: 1. 1937.
7. Blackfan, K. D., and S. B. Wolbach: *J. Pediat.* 3: 679. 1933.

SENSITIVITY TO ESTROGEN STUDIED BY MEANS OF EXPERIMENTALLY INDUCED MATING RESPONSES IN THE FEMALE GUINEA PIG AND RAT[1]

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IT IS GENERALLY RECOGNIZED that the response of an organism to estrogenic treatment is influenced not only by the quality and quantity of the substance used but also by the sensitivity or threshold to the hormone. Coward and Burn (1), Marrian and Parkes (2), and D'Amour and Gustavson (3) postulate that differences in the extent of the vaginal reaction to equal doses of estrogen are attributable to individual differences in sensitivity. The importance of age in the degree of response to estrogenic treatment is apparent from the observation (4) that the menstrual threshold to ovarian follicular hormone is high in immature rhesus monkeys and from the observation (5) that the responsiveness of the perineum in adult baboons is more marked than in prepuberal animals. Wiesner (6) states that, due to the absence of reactivity to estrogen, cornification of the vagina and enlargement of the uterus are not detectable within the first few days of post-natal life even when multiples of the physiological doses for pubescent rats are given. Duszynska (7) reports that a seasonal fluctuation in the vaginal reaction was found in castrated albino mice which received monthly injections of estrone, the response being more than three times as great in May as in November. Emmens (8) and Waterman (9) were not able to confirm Duszynska's observation, but did find an undoubted time-to-time variation in sensitivity to estrone in the vagina of mice. Susceptibility of the genital organs to estrogenic substances was reported to be inconstant in different strains of mice used by van Gulik and Kortweg (10).

Differences in threshold have also been postulated from the results of investigations on the behavioral response to estrogenic treatment. Wiesner and Mirskaia (11) regarded the failure of some of their spayed mice to exhibit the mating reflex, following an injection procedure which caused mating in a majority of cases, as being due to a high threshold for mating. Boling, Young, and Dempsey (12) assumed that certain guinea pigs which displayed abnormally short heat periods or no heat periods prior to ovariectomy possessed a low sensitivity to hormonal stimulation. After ovariectomy these animals did not respond to the estrogen-progesterone treatment which is normally effective, but they were brought into heat when twice the usual conditioning quantity of estrogen was given. Evidence that variation in the threshold to estrogen affects the occurrence of estrus in the rat is given by Hemmingsen (13), Hemmingsen and Krarup (14), Boling and Blandau (15), and Boling, Blandau, Rundlett, and Young (16).

As far as the behavioral responses are concerned the conditions under which sensitivity to estrogen varies and the factors which might account for differences

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are largely unknown. Evidence that seasonal or nutritional factors are involved is conflicting (17). On the other hand, correlation of age with differences in sensitivity is more certain. The statement by Hammond (18) that the heat period of the cow is slightly longer than that of the heifer may be significant. Indirect evidence is also provided by the observation that lambs have shorter estrous periods than older ewes, that there is no significant change between the 2nd and 8th years of age and that a a slight decrease is shown by aged ewes (19, 20). Most convincing of the data based on the length of the estrous period are those obtained from the guinea pig (21). It was found that members of this species less than 30 days of age are not as sensitive to estrogen as older animals, that the low threshold which is attained about the 30th day is maintained for at least a year, and that neither the acquisition of this sensitivity nor its maintenance is dependent on the ovaries. The present communication contains a report of an extension of these observations and some relevant data from a study of the rat.

METHODS

The sensitivity of guinea pigs and rats of different ages to the conditioning action of estrogen and the influence of the ovaries on the development of sensitivity were investigated by means of the estrous response. This response can be obtained from 80% or more of spayed adult guinea pigs after an injection of 10 R.U. of estradiol benzoate has been followed 48 hours later by 0.1 I.U. of progesterone (22, 23). For spayed adult rats, 10 R.U. of estradiol benzoate followed 48 hours later by 0.4 I.U. of progesterone is sufficient (15). When animals do not respond to these treatments, the failure may be due to low sensitivity to estrogen. The possibility can be tested by increasing the quantity of injected estrogen (12, 16). In most of the experiments described below subcutaneous injections of estradiol benzoate and progesterone[2] were made into guinea pigs from 7 days to 24 months of age and rats from 20 days to 10 months of age all of which had been spayed within 24 hours of birth. In order to determine whether the presence of the ovaries affects the development of sensitivity, other animals were ovariectomized when they were 30 and 85 days of age and injected 2 to 5 days later. To exclude the possible effects of previous injections of hormones, no animal was used in more than one experiment.

Beginning not more than 2 hours after the injection of progesterone and continuing at hourly intervals for not less than 12 hours the animals were tested by manual stimulation on the perineal region and back in order to determine whether the copulatory response (24, 25) could be elicited. Although this response is a sign of heat in sexually mature guinea pigs and rats, the authors are not certain that young individuals from which the response can be elicited would mate even if it were physically possible. Nevertheless, it was considered sufficient to use the response as a measure of sensitivity to estrogen.

Special handling of the animals was necessary only in the case of guinea pigs which were younger than 15 days at the time of injection. Lordosis can be elicited from newborn guinea pigs for several hours after birth and some animals respond intermittently for 3 or 4 days, provided they are covered by the mother (26). To avoid mistaking this lordosis for the response known to be induced by estrogen and progesterone, the young animals that were injected between the 2nd and 7th days were separated from the mothers immediately after birth and fed from a bottle.

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[2] Estradiol benzoate under the trade-name Progynon-B and progesterone under the trade-name Proluton were supplied through the courtesy of Dr. Erwin Schwenk of The Schering Corporation, Bloomfield, N.J.

Tests of sensitivity were also made on a group of animals from which the uterus and upper vagina were removed at the time of ovariectomy. The potency of the hormones used in each series of injections was determined by the simultaneous treatment of adult spayed animals with the minimal effective dose. All doses of hormones were dissolved in 0.5 cc. of corn oil except those administered to young guinea pigs, 2 to 7 days of age. The doses given these animals were dissolved in 0.25 cc. of corn oil.

RESULTS

The data bearing on the length of the experimentally induced estrus and the length of the interval between the injection of progesterone and the beginning of heat (the latent period) are summarized in tables 1 and 2. It is clear that guinea pigs less than about 30 days of age do not respond to the conditioning action of estrogen

TABLE 1. SENSITIVITY OF OVARIECTOMIZED GUINEA PIGS OF DIFFERENT AGES TO ESTRADIOL BENZOATE

Age at time of estrogen injection (days)	Hormone treatment		Number of animals	Number responding	Length of latent period		Length of heat period	
	Estradiol benzoate (R.U.)	Progesterone (mg.)			Average (hrs.)	Range (hrs.)	Average (hrs.)	Range (hrs.)
Animals ovariectomized within 24 hours after birth								
2–6	250[1]	0.4	5	0	∞	—	0.0	—
15	10	0.1	5	1	5.5	—	4.0	—
15	20	0.1	5	1	6.0	—	6.0	—
15	40	0.1	5	1	5.5	—	5.0	—
15	80	0.1	5	3	7.7	5.5–9.5	5.7	4.5–6.5
20	10	0.1	8	4	4.5	4.0–6.0	4.8	3.5–6.0
20	20	0.1	8	7	5.0	3.5–6.5	4.9	2.5–8.0
30	10	0.1	14	14	3.8	3.0–4.5	7.3	6.0–9.0
14 mos.	10	0.1	9	9	3.1	2.5–4.5	8.9	5.5–10.0
24 mos.	10	0.1	4	4	4.5	3.5–6.5	8.5	6.0–10.0
Animals ovariectomized at 30 days of age								
32	10	0.1	6	6	4.3	3.5–5.0	6.3	6.0–7.0
Animals ovariectomized at 85 days of age								
90	10	0.1	14	12	3.9	3.0–5.5	6.9	5.0–8.0
Animals ovariectomized and hysterectomized at 45 days of age								
105	10	0.1	7	7	3.6	2.5–7.0	8.1	3.0–11.0

[1] 50 R.U. a day from the 2nd through the 6th day.

TABLE 2. SENSITIVITY OF OVARIECTOMIZED RATS OF DIFFERENT AGES TO ESTRADIOL BENZOATE

Age at time of estrogen injection (days)	Hormone treatment		Number of animals	Number responding	Length of latent period		Length of heat period	
	Estradiol benzoate (R.U.)	Progesterone (mg.)			Average (hrs.)	Range (hrs.)	Average (hrs.)	Range (hrs.)
Animals ovariectomized within 24 hours after birth								
20	10	0.4	18	4	6.3	5.0–8.9	7.3	5.0– 9.0
30	10	0.4	6	6	3.7	3.0–5.5	10.1	8.5–12.0
10 mos.	10	0.4	6	5	5.0	3.5–6.0	10.4	7.0–11.0
Animals ovariectomized and hysterectomized at 55 days of age								
124	10	0.4	6	6	5.6	3.5–8.0	8.3	6.5–10.0

as readily as older animals. A smaller percentage came into heat and the estrus that was displayed was of shorter average duration than in older animals. When the quantity of injected estrogen was increased the response was better in the sense that lordosis could be elicited from a larger number of animals.

The change in sensitivity appears to be most abrupt between the 7th and 20th days. Of the five animals injected with 250 I.U. of estrogen, none gave the copulatory response following the administration of progesterone on the 7th day, whereas lordosis was elicited from nearly a third of the animals injected with smaller quantities of estrogen on the 15th day and by the 20th day 11 of 16 responded.

The change in sensitivity is independent of ovarian action because the response of the animals spayed at birth and injected on the 30th day was not different from that of the animals ovariectomized on the 30th day and injected 2 days later. Nor is the maintenance of a normal threshold dependent on the ovaries because the guinea pigs spayed within 24 hours after birth and injected for the first time 24 months later responded as readily as those whose ovaries were not removed until the 85th day by which time cyclic activity had begun. Sensitivity was not affected by hysterectomy.

Despite the greater immaturity of the newborn rat, the development of sensitivity to estrogen as measured in these experiments is similar to that in the guinea pig. Four of 18 animals in which the injections were started on the 20th day responded to manual stimulation, but all of 6 animals injected with estrogen on the 30th day displayed responses which were identical with those seen in older animals. As with the guinea pig, removal of the ovaries and uterus was without effect.

DISCUSSION

Exclusion of the ovaries as the source of stimulation for the development of sensitivity to estrogen as measured by the ability to elicit the copulatory response has eliminated the first organ to be thought of as controlling this phase of sexual differentiation in the guinea pig and rat. The result was unexpected, nevertheless, if Wiesner's observation can be confirmed, it is consistent with his conclusion that in the rat the reactivity of the vagina and uterus to estrogen develops gradually during the first 14 days of extrauterine life and is independent of ovarian action. The result is also consistent with the recent discovery that the injection of estradiol dipropionate into prepuberal female rats prevents, rather than accelerates, the acquisition of a normal sensitivity to this hormone (Wilson and Hamilton, unpublished data). Such being the case, it becomes of interest to seek clues as to the factors that might be involved in the development of sensitivity in the female during the first part of the prepuberal period.

The rôle of the other endocrines has not received systematic attention. To the extent that the few cases which have come under observation can be considered indicative, there is no evidence that the gonadotropic activity of the pituitary is involved. Female guinea pigs (27) and rats (16) have been encountered in which a normal ovarian condition, assumed to be the expression of a normal hypophyseal-gonadal relationship, existed concomitantly with a low sensitivity to estrogen. Conversely, female rats have been observed in which there was no evidence for a low sensitivity to estrogen despite the circumstance that the failure of luteinization and ovulation suggested deficient gonadotropic action (16). Doubt that the adrenals are involved is suggested by the observation that adrenalectomized rats which ultimately died of adrenal insufficiency mated soon after the operation (28).

The most interesting suggestion advanced thus far is that the development of sensitivity to estrogen is a phase of sexuality that is genetically determined in the sense that extra-gonadal factors having a genetic basis are responsible (6). This hy-

pothesis is supported, not only by the result from Wiesner's independent investigation in which different methods were employed, but also by the data presented above. It is also basically similar to the conclusion reached by Moore (29) that sexual differentiation of the ducts, gonads and phallus is controlled by sex-determining factors rather than by gonad-secreted hormones.

SUMMARY AND CONCLUSIONS

The development and maintenance of sensitivity to estrogen in female guinea pigs and rats have been studied by means of experimentally induced mating responses following injections of estradiol benzoate and progesterone into spayed animals. The hormones were administered to animals ranging in age from 7 days to 24 months. Possible influences from the ovaries and uterus were investigated.

In the female guinea pig the sensitivity to estrogen increases greatly during the first month of life. Although 7-day-old animals are insensitive to large quantities of estrogen, by about the 30th day guinea pigs attain an estrogen threshold comparable to that of adults. This threshold is maintained at a low level from the 30th day through at least the 24th month. The attainment and maintenance of this low level are independent of ovarian and uterine influences.

The female rat attains a high sensitivity about the 30th day and maintains it through at least the 10th month. Removal of the ovaries and uterus is without effect.

REFERENCES

1. Coward, K. H., and J. H. Burn: *J. Physiol.* 63: 270. 1927.
2. Marrian, G. F., and A. S. Parkes: *J. Physiol.* 67: 389. 1929.
3. D'Amour, F. E., and R. G. Gustavson: *J. Pharmacol. & Exper. Therap.* 40: 473. 1930.
4. Allen, E., A. W. Diddle, T. H. Burford and W. U. Gardner: *Am. J. Physiol.* 117: 381. 1936.
5. Gillman, J.: *S. Afr. J. Med. Sci.* 3: 66. 1938.
6. Wiesner, B. P.: *J. Obst. & Gynaec. Brit. Emp.* 41: 867. 1934 and 42: 8. 1935.
7. Duszynska, J.: *Nature* 142: 673. 1938.
8. Emmens, C. W.: *Nature* 143: 476. 1939.
9. Waterman, L.: *Acta Brevia Neerl.* 10: 109. 1940.
10. van Gulik, P. J., and R. Kortweg: *Am. J. Cancer* 38: 506. 1940.
11. Wiesner, B. P., and L. Mirskaia: *Quart. J. Exp. Physiol.* 20: 273. 1930.
12. Boling, J. L., W. C. Young and E. W. Dempsey: *Endocrinology* 23: 182. 1938.
13. Hemmingsen, A. M.: *Skand. Arch. Physiol.* 65: 97. 1933.
14. Hemmingsen, A. M., and N. B. Krarup: *Danske Videnskab. Selskab. Biol. Meddel.* 13: no. 8, 1. 1937.
15. Boling, J. L., and R. J. Blandau: *Endocrinology* 25: 359. 1939.
16. Boling, J. L., R. J. Blandau, B. Rundlett and W. C. Young: *Anat. Rec.* 80: 155. 1941.
17. Young, W. C.: *Quart. Rev. Biol.* 16: 311. 1941.
18. Hammond, J.: *The physiology of reproduction in the cow.* Cambridge. 1927.
19. McKenzie, F. F., and R. W. Phillips: *Proc. Am. Soc. Animal Prod. Bull.* no. 138. 1930.
20. McKenzie, F. F., and C. E. Terrill: *Univ. Missouri Coll. Agric. Res. Bull.* no. 264. 1937.
21. Wilson, J. G., and W. C. Young: *Anat. Rec.* 76 (suppl. 2): 58. 1940.
22. Dempsey, E. W., R. Hertz and W. C. Young: *Am. J. Physiol.* 116: 201. 1936.
23. Collins, V. J., J. L. Boling, E. W. Dempsey and W. C. Young: *Endocrinology* 23: 188. 1938.
24. Young, W. C., E. W. Dempsey, C. W. Hagquist and J. L. Boling: *J. Lab. & Clin. Med.* 23: 300. 1937.
25. Blandau, R. J., J. L. Boling and W. C. Young: *Anat. Rec.* 79: 453. 1941.
26. Boling, J. L., R. J. Blandau, J. G. Wilson and W. C. Young: *Proc. Soc. Exp. Biol. Med.* 42: 128. 1939.
27. Young, W. C., E. W. Dempsey, H. I. Myers and C. W. Hagquist: *Am. J. Anat.* 63: 457. 1938.
28. Tobin, C. E.: *Anat. Rec.* 76 (suppl. 2): 55. 1940.
29. Moore, C. R.: *Physiol. Zool.* 14: 1. 1941.

INFLUENCE OF AGE AND PRESENCE OF THE OVARIES ON REPRODUCTIVE FUNCTION IN RATS INJECTED WITH ANDROGENS[1]

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THE DEVELOPMENT OF REPRODUCTIVE FUNCTION in female rats is materially altered by the injection of testosterone compounds during the first weeks of postnatal life (1–4). When these animals reach adulthood they show abnormalities in several features of reproductive structure and function. They do not display cyclic mating behavior; ovulation and corpus luteum formation do not occur; the uterus differentiates atypically and is not normally responsive to estrogen. When such animals are spayed and injected with estrogen and progesterone estrous behavior is not induced even with 10 times the quantity of hormone (3) which causes heat in control animals.

It was concluded from these studies that testosterone propionate in doses from 3 to 36 mg. is effective in suppressing reproductive function when the injections are begun soon after birth. On the other hand, these androgens when given to sexually mature female guinea pigs (5) and rats (1, 6) caused only a temporary arrest of estrous cycles with no known permanent morphological changes except an enlargement of the clitoris noted by Ball (6). Apparently, between birth and sexual maturity there is a change in the degree of susceptibility of the animals to the action of androgen. In order to estimate the approximate age at which this change occurs and to ascertain if any ovarian influence is involved, testosterone propionate has been injected into intact and spayed female rats of different ages.

METHODS

The results obtained from the administration of androgen beginning within 24 hours of birth have been reported (3). In the present experiment the ages selected for the beginning of injections were the 5th, 10th, 15th, 20th, 30th, 40th and 120th days of life. The androgenic treatment consisted of 36 mg. of testosterone propionate[2] injected subcutaneously in doses of 3 mg. in 0.05 cc. of peanut oil three times weekly for 4 weeks.

Determinations of the extent of development of the reproductive function were made 2 to 4 months after the last injection. Tests for the occurrence of estrus were as follows. All females were confined with normal males for 2 weeks. Those which be-

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[2] Testosterone propionate, under the trade-name Perandren, was furnished through the courtesy of Ciba Pharmaceutic Products, Inc., Summit, N. J.

came pregnant were not subjected to further examination or experimentation. The remaining animals were examined for signs of heat (7, 8) at 2-hour intervals for 18 days and nights. In addition to the above tests for estrus, vaginal smears were made from animals in each group for 11 days.

Following the 18-day period of observation, the ovaries were removed from 23 animals, weighed, and at least one from each pair prepared for microscopic examination. Uterine biopsies were taken from 17 individuals in order to detect any direct effects of the androgenic treatment as well as to note signs of stimulation by endogenous estrogen.

Seven of the spayed animals were used to determine the responsiveness of the uterus to injected estrogen. Five weeks after ovariectomy a biopsy was taken from the middle of the right uterine horn. Four to 5 days later, 100 R.U. of estradiol benzoate[3] were injected and after 72 hours a biopsy was taken from the middle of the left uterine horn. The 2 specimens were compared with regard to cross-sectional area and structure.

The reactivity of the mechanism responsible for estrous behavior was tested in 7 spayed rats by 3-series of injections. The first consisted of 10 R.U. of estradiol benzoate followed 48 hours later by 0.4 mg. of progesterone.[3] The second series, made a week later, consisted of 100 R.U. of estradiol benzoate followed in 48 hours by 0.4 mg. of progesterone. Another week elapsed before a third series was given, composed of 4 daily injections of 100 R.U. of estradiol benzoate followed on the fifth day by 0.4 mg. of progesterone. After each series of injections the rats were examined at hourly intervals for 15 hours for copulatory responses.

In the earlier communication (3) a decreased sensitivity to injected estrogen was reported for female rats which had been treated with androgen during early postnatal life. The possibility was suggested that this lowered responsiveness might have been caused by an over-stimulation by estrogen from the animal's ovaries resulting in a condition of constant estrus. To determine this point 9 females were spayed and treatment with testosterone propionate was begun within 24 hours after birth. As in the other 7 groups, these animals received 36 mg. of hormone in 12 injections over a period of 4 weeks. When they reached an age of approximately 110 days, the responsiveness of the uterus and the estrous behavior mechanism to estrogen was tested by means of the procedures described above.

EXPERIMENTAL

The extent to which animals receiving temporary treatment with male hormone at various ages displayed estrous behavior upon reaching adulthood is shown in table 1. Injections started on the 5th or 10th day of postnatal life prevented the normal occurrence of estrous cycles later in life. Occasionally certain individuals showed a low-grade, non-cyclic type of behavior when tested by manual stimulation, but they did not tolerate mounting by a male rat. Constant cornification was found in the vaginal smears of 12 animals which were examined.

Estrous behavior was not abolished by injections of testosterone propionate which were begun on the 15th, 20th, 30th, 40th and 120th days except in 2 animals. Of the 9 females in which injections were begun the 15th day and which were subsequently placed with males for two weeks, 6 became pregnant and 4 bore litters. When the 3 animals which did not become pregnant were placed under continuous observation for 18 days, they exhibited 3 to 4 periods of apparently normal heat at

[3] Estradiol benzoate (Progynon-B) and progesterone (Proluton) were made available through the courtesy of Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J.

4 or 5 day intervals. When treatment was begun later than the 15th day the results were similar. Of the 18 females allowed to remain with males for 2 weeks, 12 became pregnant and 7 bore litters. Four of the 6 animals in which pregnancy did not occur came into heat every 4 or 5 days during the 18-day period of observation. The remaining 2 animals failed to show any sexual behavior and the vaginal smears revealed a tendency toward constant cornification. The fact that both rats were from the group

TABLE 1. ESTROUS BEHAVIOR IN ADULT INTACT FEMALE RATS PREVIOUSLY GIVEN TESTOSTERONE PROPIONATE

Age at the beginning of androgenic treatment	Number of animals	Estrous behavior		
		Normal	Abnormal	
			Low grade, non-cyclic	Absent
days				
5	10	0	8	2
10	8	0	2	6
15	9	9	0	0
20	4	4	0	0
30	5	5	0	0
40	4	2	0	2
120	5	5	0	0
Adults, untreated controls	10	9	0	1

TABLE 2. OVARIAN WEIGHT AND CORPUS LUTEUM FORMATION IN RATS PREVIOUSLY GIVEN TESTOSTERONE PROPIONATE

Age at the beginning of androgenic treatment	Number of animals	Average weight of both ovaries	Number of animals in which corpora lutea had formed
days		*mg.*	
5	10	33.9	0
10	4	28.5	0
15	3	71.7	3
20	2	66.0	2
30	2	(not weighed)	2

first treated on the 40th day is not considered significant. A small number of animals which are abnormal in this respect is frequently found in healthy colonies (9).

Certain conditions in the ovary were correlated with the extent to which estrous behavior was displayed. Ovaries from animals in which treatment was begun the 5th or 10th day of life were small and contained no corpora lutea (table 2). Many normal-appearing follicles were present, ranging in size from the early vesicular stages to those at which the pre-ovulatory swelling begins (10). Numerous follicles were found which exceeded this size, but they usually showed degenerative changes such as atresia or absence of the ovum and disintegration of the stratum granulosum. Ovaries from females which received the first injection of androgen on the 15th day of life or later were comparable in weight to those from untreated rats (3) and showed normal development of the follicles and corpora lutea.

The cross-sectional area of the uterine horns from rats given androgen beginning the 5th or 10th day was much smaller than that from animals in which treatment was begun later (table 3). In addition, squamous metaphasia of the mucosal epithelium had

occurred in 7 of 11 animals. The extent of modification varied from a transformation which involved the entire lining to one which affected only scattered areas. Relatively few endometrial glands were present and these appeared to have been inactive because there was no accumulation of fluids; the lumina of the glands and of the uterus as a whole were extremely small. Pyosalpinx of the type described by Bradbury (4) and the authors (3) were found in 12 of 16 individuals. Androgenic injections be-

TABLE 3. SIZE AND STRUCTURE OF THE UTERUS IN INTACT RATS PREVIOUSLY GIVEN TESTOSTERONE PROPIONATE

Age at the beginning of androgenic treatment	Number of animals	Cross-sectional area	Average number of glands per cross-section	Condition of the epithelium		
				meta-plastic	hypo-plastic	normal
days		*sq. mm.*				
5	8	2.0	2	6	2	0
10	3	1.9	2	1	2	0
15	2	5.5	8	0	0	2
20	1	5.1	6	0	0	1
30	3	5.9	6	0	0	3

TABLE 4. THE RESPONSE OF THE UTERUS TO INJECTED ESTROGEN AFTER EARLIER TEMPORARY TREATMENT WITH TESTOSTERONE PROPIONATE

Age at the beginning of androgenic treatment	Number of animals	Cross-sectional area before injection of estrogen	Cross-sectional area after injection of estrogen	Percentage increase
days		*sq. mm.*	*sq. mm.*	
5	2	1.17	1.82	56
10	2	1.02	1.76	72
15	3	1.12	3.45	208
1 (spayed at birth)	4	1.20	1.95	62
adults, untreated (spayed at birth)	8	0.34	2.37	580
adults, untreated (spayed as adults)	8	1.74	4.94	184

ginning the 15th day or later did not produce any of these effects; 3 to 4 months after the last injection of testosterone propionate the uterine structure was indistinguishable from that in untreated adult females.

The uterine tissues in the animals treated with androgen beginning the 5th or 10th day were generally metaplastic and atrophic, yet there were indications of androgenic as well as a degree of estrogenic stimulation. The average cross-sectional area of uterine horns removed from eight 4-month-old animals spayed within 24 hours of birth was 0.34 sq. mm. The average area was somewhat larger, 1.2 sq. mm., in four 4-month-old animals spayed within 24 hours of birth and immediately given androgen. The difference is attributed to the action of the injected androgen. On the other hand, the cross-sectional area of the uterine horns from animals injected beginning the 5th or 10th day, but not ovariectomized until 3 to 4 months later, was still larger, averaging 2.3 and 1.9 sq. mm., respectively. The larger size of the uterine tissues in the latter cases is thought to have been produced by ovarian secretions. Additional evidence for this opinion comes from the observation that the areas of the epithelium of the glands and surface which had not undergone metaplasia were more nearly normal than those in uteri from spayed, androgen-treated females.

The response of the uterus to injections of estrogen was considerably less in animals treated with male hormone beginning on the 5th or 10th days of life than it was in those in which treatment was delayed until the 15th day or in those which were not injected with androgens but were spayed either at birth or as adults (table 4). The lack of uterine response to estrogens cannot be ascribed to an over-stimulation by ovarian secretions since the uteri from animals spayed within 24 hours of birth and treated with androgen were also resistant to later administration of estrogen. It appears, therefore, that the poor response was caused by the androgenic treatment.

Abnormalities in ovarian and uterine structure similar to those described above are illustrated in plates I, II and III of our previous report (3).

The sensitivity of the mechanism responsible for estrus is assumed to have been

TABLE 5. SENSITIVITY OF THE ESTROUS BEHAVIOR MECHANISM TO INJECTED ESTROGEN IN ANIMALS TREATED EARLIER WITH TESTOSTERONE PROPIONATE

Age at the beginning of adrogenic treatment	Number of animals	Number of females displaying mating behavior after different doses of estradiol benzoate followed by 0.4 mg. of progesterone		
days		10 R.U.	100 R.U.	400 R.U.
5	3	0	1	3
10	4	0	1	2
1	5	0	1	3
(spayed at birth) adults, untreated	5	5	5	5
(spayed at birth) adults, untreated (spayed as adults)	18	17	18	18

but little, if any, affected in females treated with androgen beginning on the 15th day of life or later because most of these animals mated or at least displayed cyclic estrous periods. Injections begun the 5th or 10th days, on the other hand, had a definitely harmful action. When animals in these groups were spayed and subjected to different doses of estrogen followed by progesterone, a majority were brought into heat only when the largest quantity of estrogen was used (table 5). As in the case of the uterus, the changes cannot be ascribed to over-stimulation by endogenous estrogens because females spayed at birth and given androgen beginning the first day were also insensitive to injected estrogen.

DISCUSSION

Some factors that may be involved in the impairment of reproductive function by postnatally-administered testosterone propionate are discussed elsewhere (3). An effect on the hypophyseal-gonadal relationship is indicated by the fact that in most animals luteinization did not occur although follicular development was normal up to the point at which the pre-ovulatory swelling usually begins. Bradbury (4) has since shown that the pituitary must be a site of primary dysfunction. When ovaries from such animals were transplanted into normal females or when APL extracts were injected, ovulation and luteinization followed. A second important effect of androgenic treatment was the alteration of the normal threshold to estrogenic stimulation in such a way that the tissues of the uterus and the mechanism responsible for estrous behavior were not responsive to the quantity of estrogen which is usually effective in untreated animals.

Results from the present experiments confirm these conclusions, direct attention to the importance of the age of the animal at the time of initiation of treatment and

demonstrate that the abnormalities of structure and function are not induced by action of the ovaries. When a total dose of 36 mg. of testosterone propionate was administered an abrupt change in the degree of susceptibility was indicated as occurring between the 10th and 15th days of the postnatal period. By every measure of reproductive function that was used, injections of this particular dose begun the 5th or 10th day were as harmful as those started the 1st day (3). On the other hand, treatment begun the 15th day or later caused no permanent defects of the types studied.

The cause of the widespread defects in the reproductive system of female rats treated postnatally with androgen remains unknown. The suggestion has been made that 'masculinization' of the female pituitary is responsible for the ovarian dysfunction (4, 11, 12), but in unpublished studies we have observed that early postnatal administration of estrogens produces structural and functional abnormalities that are somewhat similar to and, in some instances, more severe than those obtained with androgens.

The discovery that a change in susceptibility to androgenic treatment takes place between the 10th and 15th days is of interest in connection with the results from two independent series of observations. Wiesner (13) observed that the uterus and vagina of young rats did not respond to estrogenic stimulation until about the 15th day. Wilson and Young (14) were not able to induce heat in young guinea pigs and rats by injecting estrogen and progesterone at an age earlier than 15 days. It may be that the age at which injected androgen no longer exerts a permanently harmful action is the same as that at which female rats attain a lower sensitivity to estrogen. Notwithstanding, these more recent observations reinforce the conclusion that about the 15th day of life the organism is undergoing a change which is of major importance for responsiveness to sex hormones.

SUMMARY

A standard dose of 36 mg. of testosterone propionate was administered in 12 injections over a period of 4 weeks to female rats of different ages.

Injections beginning the 15th, 20th, 30th, 40th or 120th days of age caused no permanent impairment of reproductive function. After reaching maturity females subjected to this treatment ran normal estrous cycles, mated and bore litters. When the injections were started the 5th or 10th day, the animals later showed permanent defects in several features of reproductive structure and function. They failed to display cyclic estrus. Ovulation and luteinization did not occur. The uteri differentiated abnormally and responded poorly to injected estrogen. Ovariectomized animals of this type were not normally reactive to estrus-inducing hormones. A similar lack of responsiveness was observed in female rats spayed at birth and treated with androgen. The decreased sensitivity to estrogen, therefore, was not brought about by ovarian secretions.

REFERENCES

1. Mazer, M., and C. Mazer: *Endocrinology* 24: 175. 1939.
2. Selye, H.: *Endocrinology* 27: 657. 1940.
3. Wilson, J. G., W. C. Young and J. B. Hamilton: *Yale J. Biol. & Med.* 13: 189. 1940.
4. Bradbury, J. T.: *Endocrinology* 28: 101. 1941.
5. Boling, J. L., and J. B. Hamilton: *Anat. Rec.* 73: 1. 1939.
6. Ball, J.: *J. Comp. Psychol.* 29: 151. 1940.
7. Hemmingsen, A. M.: *Skand. Arch. Physiol.* 65: 97. 1933.
8. Blandau, R. J., J. L. Boling and W. C. Young: *Anat. Rec.* 79: 453. 1941.
9. Boling, J. L., R. J. Blandau, B. Rundlett and W. C. Young: *Anat. Rec.* 80: 155. 1941.
10. Boling, J. L., R. J. Blandau, A. L. Soderwall and W. C. Young: *Anat. Rec.* 79: 313. 1941.
11. Pfeiffer, C. A.: *Am. J. Anat.* 58: 195. 1936.
12. Pfeiffer, C. A.: *Anat. Rec.* 75: 465. 1939.
13. Wiesner, B. P.: *J. Obst. & Gynaec. Brit. Emp.* 41: 867. 42: 8. 1934 and 1935.
14. Wilson, J. G., and W. C. Young: *Endocrinology* 29: 779. 1941.

CONDITIONS INFLUENCING THE COURSE OF STEROID HORMONE ANESTHESIA[1]

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IT HAS BEEN FOUND RECENTLY that steroid hormones produce anesthesia when injected intraperitoneally or intravenously. If they are injected subcutaneously they do not act in this manner probably because in the latter case, the rate of their absorption is comparatively slow (1). Up to the present, all observations concerning this action of the steroids are in agreement with what has been called the 'fundamental law of steroid hormone anesthesia,' namely, that all compounds having a steroid hormone action are capable of producing anesthesia while no compound devoid of hormone action possesses this power (1–4). It has also been established that following repeated intraperitoneal administration of anesthetic steroids, the

TABLE 1. EFFECT OF SEX ON PROGESTERONE ANESTHESIA IN RATS.

Group	No. Rat	Weight gm.	Maximum Anesthesia[1]	No. reaching max.	Remarks
Normal male	10	131	+	1	Rest normal
Male castrates pre-treated for 3 weeks with 2 mg. of methyl testosterone daily subcutaneously	10	135	−	−	All normal
Male castrates	9	113	+++	4	3 trace or +; 3 normal
Normal females	10	105	+++	10	−
Female castrates	10	109	+++	8	2++
Female castrates pre-treated for 3 weeks with 2 mg. of methyl testosterone daily subcutaneously	10	123	−	−	All normal

[1] Stages of anesthesia, +++—deep anesthesia in which the righting reflex has disappeared; ++—light anesthesia in which the righting reflex is still present; +—first stage of anesthesia—but no excitation; trace—early part of first stage.

animal body develops a resistance against their narcotic action (2). Systematic studies have been conducted concerning a variety of factors (nephrectomy, hepatectomy, hypophysectomy, adrenalectomy and various drugs) capable of influencing the course of steroid hormone anesthesia (2). However, little is known about the influence of the sex glands, although attention has been drawn to the fact that females are more sensitive than males to the narcotic action of all the steroid hormones so far examined. There appeared to be two possible explanations of this sex-conditioned difference in sensitivity. Either the somatic cells of the male possess an innate

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TABLE 2. EFFECT OF VARIOUS AGENTS ON PROGESTERONE ANESTHESIA IN RATS

Treatment	No. and sex of rats		Wt. gm.	Average anesthesia			Dead
	♀	♂		.5	1.5	5 hrs.	
Progesterone, 3 mg.	4	3	41	++	++	0	1
Insulin, 0.5U+progesterone, 3 mg.	3	4	39	++	+++	+++	1
Progesterone, 3 mg.	4	6	35	++	+++	0	2
Progesterone, 3 mg.+cold (1 hr. at −6°C)	5	5	35	+++	+++	+++	9
Progesterone, 3 mg.+heat (1 hr. at 38°C)	6	4	33	+++	+++	+	3
Adrenalin, 0.1 cc. of 1:1000 +progesterone, 3 mg.	5	5	37	trace	++	—	—
				.5	2.5	3.5 hrs.	
Glucose intravenously, 2 cc. 5.5%+progesterone[1] 10 mg. ½ hr. later	3	4	61	trace	trace	+	
Saline intravenously, 2 cc. 0.9% progesterone[1] 10 mg. ½ hour later	3	3	63	+	++	+++	1
				.5	1.5	2.5 hrs.	
Progesterone, 6 mg.	3	4	69	++	++	+	—
Glucose intraperitoneally, 2 cc. 5.5%+progesterone, 6 mg.; simultaneously. 2¼–2½ hrs. later 4 cc. glucose intraperitoneally	3	5	70	trace	++	trace	—
				.6	1	2.5 hrs.	
Progesterone, 5 mg.	5	5	56	++	++	+++	—
Glucose subcutaneously, 2 cc. 10%+progesterone, 5 mg.; simultaneously	5	5	56	+	++	++	—
Saline subcutaneously, 2 cc. 0.9%+progesterone, 5 mg. simultaneously	5	5	55	+++	+++	+++	—
				.5	1	1.5	2 hrs.
Progesterone, 3 mg.	4	3	39	++	++	++	+
Glucose subcutaneously, 2 cc. 10%+progesterone, 3 mg.; simultaneously. 1½ hrs. later 2 cc. glucose subcutaneously	4	3	42	+	++	+	0

[1] Since the degree of anesthesia obtained was only + or trace after 4 mg. of progesterone, two further doses of progesterone were given between each reading recorded above, the first being 2 mg. and the second 4 mg., thus making a total dose of 10 mg.

resistance or else the endocrine secretion of the gonad influences hormone sensitivity. The fact that the relative resistance of the male develops only after puberty appears to support the latter view but in order to elucidate this phenomenon, a number of experiments had to be performed and these are the subject of the present communication.

Six groups of immature albino rats having an average weight of about 45 gm. at the beginning of the experiment were treated in the manner indicated in table 1. After a 3-week pretreatment period, an intraperitoneal injection of 8 mg. of progesterone in 0.2 cc. of peanut oil was given to all animals except the females and fe-

male castrates which, having grown somewhat more slowly than the rest, received only 7 mg.

The results summarized in table 1 clearly indicate that, in the dose given, progestrone exerted no anesthetic action in the normal males and in the methyl testosterone pretreated male castrates or females. About 50% of the untreated male castrates and all female controls and female castrates went into deep narcosis. These findings show that the male sex hormone in the quantities in which it is physiologically produced by the male gonad confers a resistance towards steroid hormone anesthesia. This can be imitated in female or male castrates by treatment with an artificial testoid substance[2] such as methyl testosterone. One might think that secretion of luteoids in the female would confer some resistance towards progesterone anesthesia; it must be borne in mind, however, that while the secretion of testoids in the male is continuous, luteoid secretion in the female is intermittent and of relatively short duration. Whether non-endocrine somatic factors play any rôle in the anesthesia resistance of males is questionable even though in this experiment, the male castrates appeared to be somewhat more resistant than females. It is possible that castration may not have produced all of its effects within the 3-week interval allowed between gonadectomy and progesterone administration.

In the course of similar experiments, it was incidentally observed that insulin administration and exposure to heat or cold increase, while treatment with adrenalin or glucose decreases the sensitivity to the anesthetic effect of progesterone. The fact that the action of isotonic saline solutions was similar, though less pronounced than that of glucose solutions casts some doubt on the specificity of the glucose action. Simple dilution of the hormone in the blood or in the peritoneal cavity could explain the action of fluid administration although it is yet to be established why glucose appears to be more active than NaCl. The fact that glucose administration some time after the injection of progesterone tends to awaken the rats is subject to the same consideration. The results of these experiments are summarized in table 2.

SUMMARY

Female rats are more sensitive to the anesthetic action of progesterone than males but this sex difference is obvious only after maturity. The normal endocrine activity of the testis is largely, if not entirely, responsible for this comparative resistance of the males since castration increases sensitivity in males but is without effect in female rats. Conversely the resistance of castrate males and females may be raised by methyl testosterone administration.

Treatment with glucose and adrenalin tends to increase, while treatment with insulin and exposure to excessive heat or cold tends to decrease the resistance of the rat to the anesthetic action of progesterone.

Expenses of this investigation were defrayed through a grant received from the Schering Corporation of Bloomfield, New Jersey. The author wishes to express her thanks to Drs. Gregory Stragnell and Erwin Schwenk for supplying the methyl testosterone and progesterone used in these experiments.

REFERENCES

1. Selye, H.: *J. Immunol.* 41: 259. 1941.
2. Selye, H.: *J. Pharmacol. and Exper. Therap.* (in press).
3. Selye, H.: *Proc. Soc. Exper. Biol. & Med.* 46: 116. 1941.
4. Selye, H.: *J. Pharmacol. and Exper. Therap.* 71: 236. 1941.
5. Selye, H.: *Nature* 148: 84. 1941.

[2] The terms "testoid" and "luteoid" are used here as synonymous with the rather cumbersome terms "male hormone-like activity" and "corpus luteum hormone-like activity" in agreement with the terminology which designates the "adrenal cortical hormone-like activity" as "corticoid" and the "follicular hormone-like activity" as "folliculoid" (5).

SERUM CHOLESTEROL IN THE RHESUS MONKEY

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BECAUSE OF THE SIMILARITY in molecular structure of the long-known cholesterol and the newer steroidal sex hormones and because of the fact that no such records exist for any primate animal, it seemed desirable to make some readings of the cholesterol level in the blood of the rhesus monkey. Since the level of the sex hormones changes with the various phases of the menstrual cycle and with pregnancy, samples of blood were taken at various times of the cycle and of pregnancy so that the stages were pretty well covered by the total number of readings from all of the animals used. A number of samples were taken from each animal.

The blood was drawn from the saphenous vein, placed in a clean test-tube, the serum separated and analyzed at a convenient time soon after collecting. The total cholesterol was determined by the colorimetric method of Bloor as adapted by Pijoan and Walter (1) to the Evelyn photo-electric colorimeter.

THE MENSTRUAL CYCLE

Twelve menstruating females were selected from the colony, more or less at random, except that an effort was made to include both ovulating and non-ovulating individuals. Otherwise all of the subjects were normal, vigorous animals whose menstrual cycles varied in length to the normal extent. In plotting the cholesterol readings, however, the cycle was reduced or expanded to one of 28 days and the reading of any given day was entered as if the cycle had been 28 days, that is, proportionately later than actually occurred if the cycle in question happened to be less than 28 days, proportionately earlier if the given cycle was longer than 28 days.

This 'synthetic' 28-day cycle was subdivided into 4 five-day periods and a final eight-day period. The average readings and the ranges for each period are tabulated on this basis in table 1. The readings ranged from 79 to 191 mg.% except for one of 55; only 5 were higher than 170 mg.%.

A study of the table fails to reveal any consistent rhythm of cholesterol level fitting into the menstrual cycle. There is, however, a considerable difference between readings for the ovulatory and the non-ovulatory cycles. In the former 63 readings yielded an average of 126($\pm$25) mg.%,[2] whereas 37 readings in non-ovulatory cycles gave an average of 111($\pm$18) mg.%. The difference between the means for the ovulatory and non-ovulatory cycles is 15 mg.% with a standard error of 4.3. The critical ratio is 3.5 indicating a satisfactory degree of statistical significance.

PREGNANCY

Thirty-three readings were made during pregnancy and these averaged 91 mg.%. The 12 readings taken from the first half of pregnancy averaged 110($\pm$26) mg.%, al-

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[2] Standard deviation.

most exactly the same as those in the non-ovulatory cycles (table 1). Twenty-one readings of the second half of pregnancy averaged only 80(±16) mg.%. The former ranged from 78 to 154 mg.%, the latter from 52 to 107 mg.%, only 3 of the latter running over 100 mg.%. The difference between the means for the first and second half of pregnancy is statistically significant, the critical ratio being 3.6.

Hyperestrinized Cases

Twenty-three blood cholesterol readings were made from 7 monkeys which had been receiving for more than two years over 100,000 R.U. of estrogen weekly in oil

TABLE 1. SERUM CHOLESTEROL DETERMINATIONS IN THE FEMALE RHESUS MONKEY

Physiologic Phase	No. of Readings	Cholesterol mg. % Range	Av.	No. of Readings	Cholesterol mg. % Range	Av.
Menstrual Cycle		Ovulatory			Non-Ovulatory	
D. 1– 5	17	101–140	122	8	92–140	117
D. 6–10	12	103–191	135	7	100–121	111
D. 11–15	9	105–155	120	9	55–121	101
D. 16–20	10	66–162	124	3	90–130	99
D. 21–28	15	86–188	128	10	103–160	120
Total	63	66–191	126	37	55–160	111
Pregnancy		First Half			Second Half	
	12	78–154	110	21	52–107	80
Hyperestrinized	23	103–212	151			

and in the form of subcutaneous implanted pellets. They were still being treated when the blood cholesterol determinations were made. These averaged 151(±24) mg.% and ranged from 103 (the only one under 125) to 212 mg.%.

DISCUSSION

A mere beginning has been made in the study of the metabolism of the steroidal hormones, the estrogens, the androgens, progesterone and desoxycorticosterone. The problem is of considerable physiological importance both theoretically and clinically. The rôle of cholesterol in relation to the newly-discovered sex steroids is not known. It is even a matter of speculation whether this stable compound is the end product of metabolism or the mother substance from which the body makes the steroidal hormones.

The former interpretation would seem to be favored by comparison of the high titer in the hyperestrinized animals (151 mg.%) with the low titer (80 mg.%) observed during the second half of pregnancy. However, it must be borne in mind that the level of cholesterol in the serum need not necessarily be an index of the cholesterol content of the entire body. Furthermore the non-ovulatory females, which presumably are producing less hormone, have a lower titer (111 mg.%) than the endocrinologically more active females (126 mg.%).

The low concentration of cholesterol in the pregnant monkeys is of interest in view of the fact that in women the blood cholesterol increases gradually until term. Boyd (2) finds an average plasma cholesterol of 181(±22) mg.% in non-pregnant women, an average of 205(±45) mg.% in pregnant women. Tyler and Underhill (3)

state that blood cholesterol at term is approximately one-third higher than during the third month of pregnancy. In the rabbit, on the other hand, blood cholesterol shows a tendency to decrease during pregnancy (4, 5). The average blood cholesterol before pregnancy and during the first trimester is 90 mg.% and falls to 60 mg.% in the second trimester, which level is maintained till the end of pregnancy (5).

Few data are available on the effect of large doses of estrogens on blood cholesterol in women. Mazer and his associates (6) find no appreciable changes in blood cholesterol in women who have received a total of 100,000 to 200,000 R.U. of estrogens in divided doses over periods of 2 to 3 months. The same is true following treatment with stilbestrol (1 mg. twice weekly (7)). A total of 9 mg. of estradiol propionate given over a period of 100 days had no influence on the blood cholesterol of rabbits (8). In birds, however, hypercholesterolemia is easily induced by estrogens (9, 10).

SUMMARY

The serum cholesterol level of 156 readings from 27 monkeys averaged 118 mg.%. One hundred readings at various times of the menstrual cycle averaged 120 mg.%, 111 for non-ovulatory, 126 for ovulatory cycles. During pregnancy the 23 readings averaged 91 mg.%, those taken in the second half of gestation, 80 mg.%; those in the first half, 110 mg.%. The blood of hyperestrinized monkeys yielded readings that averaged 151 mg.%, almost double that found in late pregnancy or the early puerperium.

We wish to thank Dr. Christopher Tietze of the Johns Hopkins University School of Hygiene for his help with the statistical evaluation of our data.

REFERENCES

1. Pijoan, M., and C. W. Walter: *J. Lab. and Clin. Med.* 22: 968. 1937.
2. Boyd, E. M.: *J. Clin. Invest.* 13: 347. 1934.
3. Tyler, M., and F. P. Underhill: *J. Biol. Chem.* 66: 1. 1925.
4. Baumann, E. J., and O. M. Holly: *Am. J. Physiol.* 75: 618. 1926.
5. Patterson, W. B., H. F. Hunt and R. E. Nicodemus. *Am. J. Clin. Path.* 8: 120. 1938.
6. Mason, C., D. R. Meranze and S. L. Israel: *J.A.M.A.* 105: 257. 1935.
7. Mazer, C., S. L. Israel and E. Ravetz: *J.A.M.A.* 116: 675. 1941.
8. Luden, J. B., M. Bruger and J. S. Wright: *Endocrinology* 28: 999. 1941.
9. Entenmann, C., F. W. Lorenz and I. L. Chaikoff: *J. Biol. Chem.* 126: 763. 1938.
10. Landauer, W., C. A. Pfeiffer, W. U. Gardner and E. B. Man: *Proc. Soc. Exper. Biol. & Med.* 41: 80. 1939.

STUDIES ON STILBESTROL

I. SOME EFFECTS OF CONTINUOUS INJECTIONS OF STILBESTROL IN THE ADULT FEMALE RAT

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THE ANNOUNCEMENT BY DODDS (1) and his associates of the high estrogenic activity of stilbestrol opened up a field of research which promised to be very fruitful. In view of the fact that the chemical structure of stilbestrol was different from that of any of the natural estrogens it seemed highly desirable and necessary to investigate the parallelism of physiological and pharmacological effects of stilbestrol and the natural estrogens. Many reports had appeared on the effects of estrone, estradiol and their esters on some of the other endocrine glands. Accordingly this work was undertaken to compare the effects of stilbestrol directly with those of natural estrogens on the pituitary, thyroid, adrenals, pancreas, ovaries, uterus, vagina, liver and kidneys of animals treated with comparable estrogenic doses of both substances.

Since the first work appeared in 1938 the volume of literature on stilbestrol has grown so rapidly that it would be impracticable here to attempt a review. A small monograph would be necessary to analyze the more than 350 papers which have been published. Suffice to say that it has been established that stilbestrol is a synthetic compound having in general the qualitative effects of the natural estrogens.

MATERIALS AND METHODS

The stilbestrol[2] used in these studies was synthesized from anise aldehyde and identified by physical and chemical means as 4:4-dihydroxy-α:β-diethylstilbene. Corn oil solutions were employed throughout.

The natural estrogen[3] employed was prepared from pregnant mares' urine as a standard routine procedure in manufacture. It was carefully standardized in terms of the international unit of estrone on castrate female rats. All solutions used were in corn oil and dosages designated in gamma equivalents of estrone. This was necessary because the natural estrogen preparation contains in addition to estrone small amounts of the other estrogens found in pregnant mares' urine.

In castrate female rats weighing 150 to 200 gm. a definite ratio was set up between the estrogenic activity of stilbestrol and estrone. When given in corn oil solution in 1 injection it was found that 0.5γ of stilbestrol in 1 cc. produced fully cornified smears in 80% of the rats treated. With the natural estrogen it required 2.5γ to produce estrus in 80% of the rats.

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[1] With the technical assistance of Miss C. M. Cushing and Mrs. A. M. Alvey.

[2] Prepared in the Squibb Development Laboratories, New Brunswick, N. J., under the direction of W. G. Christiansen.

[3] Amniotin, Squibb Biological Laboratories, New Brunswick, N. J.

On this basis a comparative study was made of the effects upon the endocrine glands and some other organs of physiological (estrus-inducing) dosages and various multiples of that dose over varying periods of time. Five rats were used in one cage at each dose level and for each time interval. One injection of 1 cc. was given daily. Vaginal smears were taken daily at the time of injection.

In all cases the pituitaries, thyroids, adrenals, pancreas, ovaries, uteri and liver were removed at autopsy. These were fixed in Bouin's solution, sections cut, stained and examined. The tissue from at least 2 animals in each cage of 5 was studied.

NORMAL FEMALE RATS

Procedure. Two series of experiments were performed. The first consisted of adult female rats receiving doses of stilbestrol of 50, 5, 1.0 and 0.5γ daily. A similar group of rats were given 250, 50, 2.5 and 1.25γ of Amniotin daily. At the end of 15, 30 and 52 days of injections one cage on each dosage level was killed. After a rest period of 20 days following the last injection the remaining cage on each dose level was killed.

In the second series a group of rats were given 250 and 100γ of stilbestrol daily and another group received 500γ Amniotin daily. A cage from each of these three dosage levels was killed at the end of 28, 49, 64 and 90 days of treatment. Twenty-three days after the last injection a cage of rats on each dose level was killed.

RESULTS

Examination of the daily vaginal smears indicated that irrespective of the dosage of either type of estrogen employed, completely cornified smears were not maintained. In fact after a few days of treatment the appearance of a fully cornified smear was sporadic and very occasional. This is in contrast to what was found in castrate female rats in similar experiments. This agrees with the work of Clauberg as reported by von Pallos (2) who states that mucification of the vaginal epithelium occurs when estrogens are administred continuously to normal female rats.

The physical condition of the animals remained satisfactory throughout the treatment with the exception that in some on the larger doses for the longest treatment period, the hair became thin. This has been reported by others following the administration of the natural estrogens. The mortality in such a large group was approximately that of a similar untreated group of the same age level.

Ovary. Most authors report a suppression of follicular activity as the result of long-continued injections of estrogens. Since we found follicles and corpora lutea in all stages of development in our normal control animals we gauged roughly the estrogenic effect by the degree of suppression of follicles. Thus if we found several fresh corpora lutea with few maturing follicles we judged the suppression to be recent. If old corpora lutea and small atretic follicles only were observed we judged the suppression to have existed for some time.

Considerable individual variation in the rats was observed. Each cage contained 5 rats and one of these might show an ovary containing only 1 large corpus luteum while another in the same cage might show 7 old corpora lutea.

In the first series with the dosage of both estrogens varying from the estrogenic dose up to 100 times that dose the ovaries showed a wide variation of response. Following treatment with the lowest doses of both estrogens there was no effect on the ovaries at the end of either 15 or 30 days of treatment and very little even after 52 days. After the rest period all ovaries in these groups were normal.

With 50γ doses of stilbestrol suppression was noticed after 15 days of treatment, persisted throughout treatment and recovery was not complete 20 days after injections were stopped.

With Amniotin at the corresponding dose level of 250γ the suppression was not as great at the end of 30 days as it was after 15 days with stilbestrol. It was complete, however, after 52 days of injections and recovery was likewise incomplete after the rest period.

In the second series with doses of Amniotin 200 times the estrogenic dose and with stilbestrol in 200 and 500 times that dose and for as long as 90 days of treatment, somewhat greater effects were produced.

After 28 days of treatment with either of the estrogens, at all of the dose levels tried, the ovaries contained corpora lutea. These tended to disappear as injections continued for 49, 64 and 90 days. After a rest period of 23 days following 90 days of treatment corpora lutea were again present.

At the end of 28 days of treatment follicles were present in all stages as well as corpora lutea indicating that more than 28 days of treatment is necessary to suppress the follicular function. The suppression is only temporary as shown by the recovery after 23 days without injection.

Where no corpora lutea were present the follicles generally were small and the stroma dense. The only sign of activity was mitosis of the granulosa cells of these follicles. The dense stroma probably indicated an excessive formation of atretic follicles.

One ovary, following 250γ of stilbestrol for 49 days, exhibited a large follicular cyst in which squamous epithelium was growing.

One ovary in each group showed after 64 days of treatment an acute inflammatory condition or abscess in the neighboring fallopian tube.

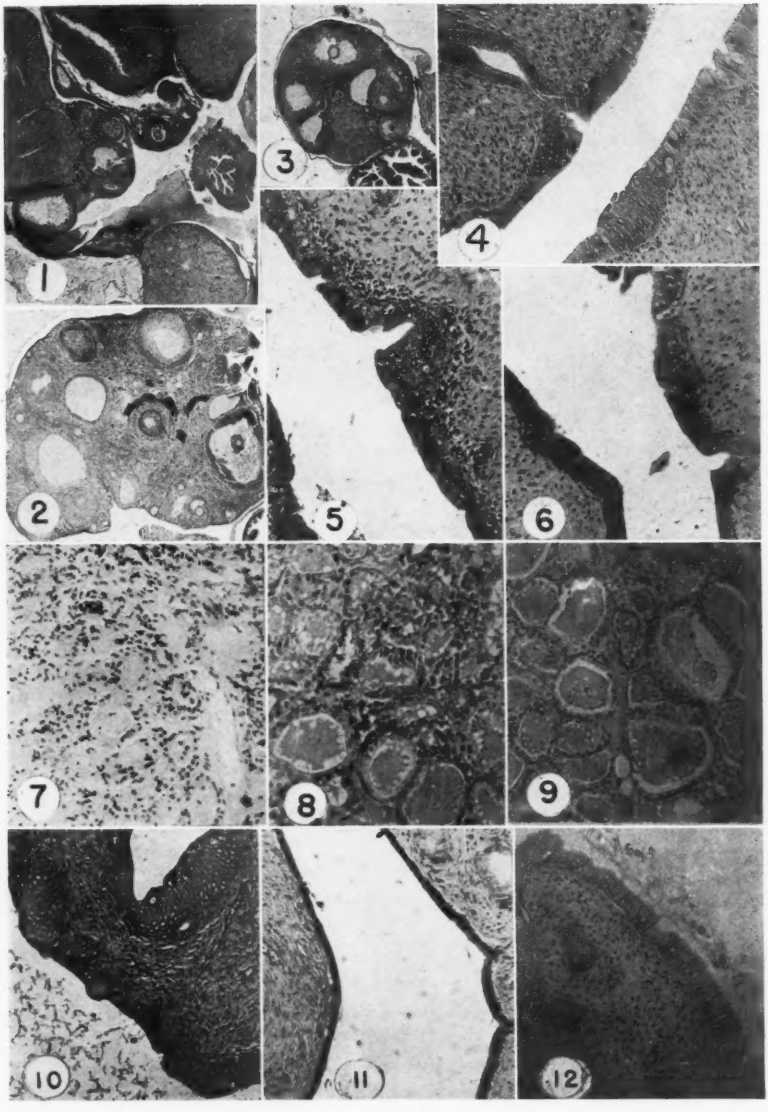
The effect of the 2 estrogens was very similar with evidence of slightly greater activity of stilbestrol even at the corresponding estrogenic dosage. The suppression of follicular activity was accomplished only after considerable periods of time and only then when several times the estrogenic dosage was administered. This effect is temporary and from the evidence accumulated, recovery would probably be complete in about 30 days after cessation of treatment.

These results are in agreement with those of Freud (3) who found that 50γ of estrone daily for 10 to 20 days had no marked effect upon ovarian structure in adult rats. Korenchevsky (4) using estradiol-3-benzoate-17-butyrate in weekly doses of 90γ did not observe marked effects on the ovaries of his rats.

In figure 1 is shown recent suppression of ovarian function. Five large corpora lutea are present with a few small follicles. This rat had received 5γ of stilbestrol daily for 15 days. In figure 2 there is one medium sized corpus luteum and several large follicles. The absence of corpora lutea and presence of many follicles in all stages is shown in figure 3. These are typical of the variation found and bear little correlation to dosage although long-continued treatment with the higher doses caused greater changes and more consistent evidence of suppression of activity.

Uterus. Long and Evans (5) have described the changes in the uterus of the rat during the normal estrous cycle. In the resting stage it is small, with a slit-like lumen, lined with columnar epithelium. As the cycle progresses it is distended by secretion and the epithelium becomes cuboidal. The secretion is discharged somewhere near the height of estrus and the uterus returns to its natural size. The epithelium is now tall, but vacuolated and the submucosa and mucosa are full of leucocytes.

Fig. 1–12. Effects of stilbestrol and Amniotin on intact female rats. Fig. 1. C. 64–4. Ovary, 5 large corpora lutea, few small follicles; 5γ of stilbestrol; 15 days; ×6. Fig. 2. C. 72–9. Ovary, 1 moderate sized corpus luteum; several large follicles; 0.5γ of stilbestrol, 15 days; ×6. Fig. 3. C. 68–3. Ovary, no corpora lutea, follicles all stages; 1γ of stilbestrol, 15 days; ×6. Fig. 4. C. 86–2. Uterus, metaplasia; note



patch of squamous epithelium growing under the columnar epithelium; 250γ of Amniotin; 52 days. ×100. Fig. 5. C. 67–2. Uterus, tall columnar epithelium with leucocytes gathered below mucosa and in lumen; 5γ of stilbestrol, 52 days then 20 days rest; ×100. Fig. 6. C. 63. Uterus, tall columnar epithelium with leucocytes beginning to enter mucosa; 50γ of stilbestrol, 52 days then 20 days rest; ×100. Fig. 7. C. 60–1. Thyroid, almost complete destruction of acinar structure; fibrosis; hyaline degeneration; 50γ of stilbestrol 15 days; ×150. Fig. 8. C. 68. Thyroid; moderate infiltration with about one-half of acini damaged; 1γ stilbestrol; 15 days; ×150. Fig. 9. C. 84. Thyroid, slight infiltration and damage; epithelium is cuboidal; 250γ of Amniotin, 15 days; ×150. Fig. 10. C. 11. Uterus, metaplasia; 0.5γ stilbestrol, 52 days; ×100. Fig. 11. C. 17. Uterus, cuboidal epithelium; 5.0γ of stilbestrol, 17 days; ×100. Fig. 12. C. 9. Uterus, secreting epithelium; 0.5γ of stilbestrol, 17 days; ×100.

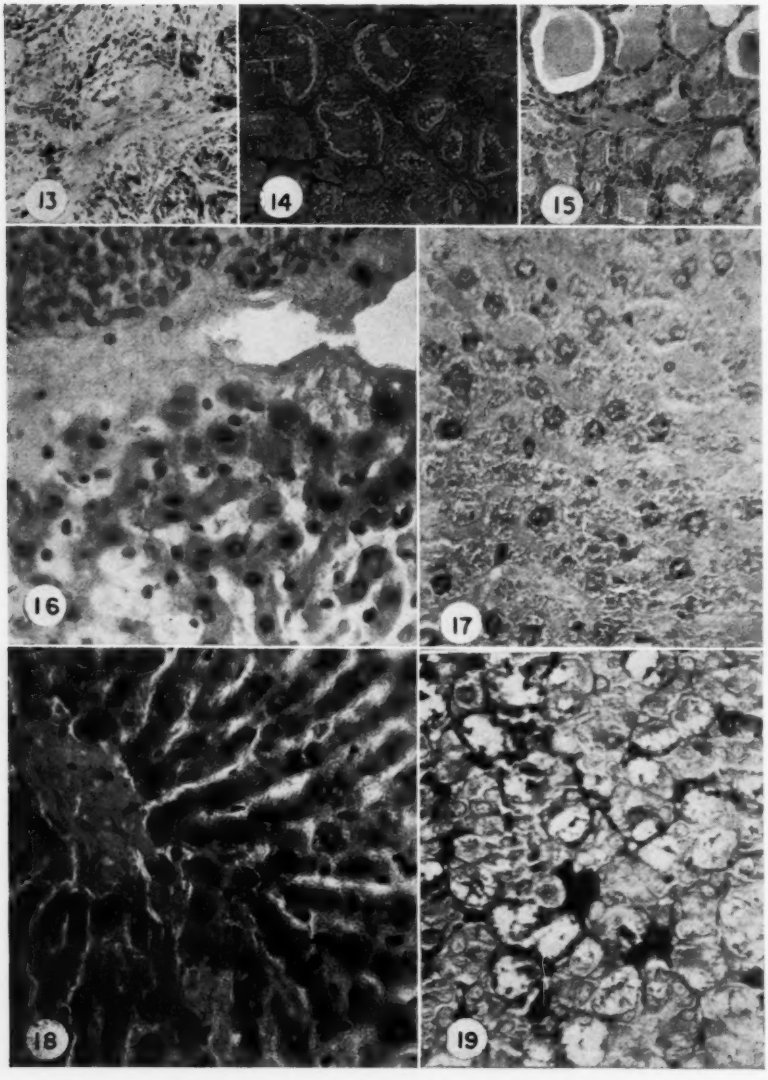


Fig. 13–19. Effects of stilbestrol in castrate female rats. Fig. 13. C. 10–4. Thyroid, heavy infiltration; fibrosis with acinar structure faintly indicated; 0.5γ of stilbestrol, 29 days; ×150. Fig. 14. C. 9. Thyroid, moderate infiltration with destruction of about ¼ of acini; 0.5γ of stilbestrol, 17 days; ×150. Fig. 15. C. 17–3. Thyroid, slight infiltration with slight damage to acini; 5.0γ of stilbestrol, 17 days; ×150. Fig. 16. C. 24. Liver, maximum damage (see text). 50γ of stilbestrol, 52 days, then 20 days rest; ×430. Fig. 17. C. 16–1. Liver, moderate damage, cell outlines blurred, vacuolization and hemorrhage; 1γ of stilbestrol, 52 days, then 20 days rest; ×430. Fig. 18. C. 18–2. Liver, very slightly damaged; 5γ of stilbestrol, 29 days; ×430. Fig. 19. C. 109. Anterior pituitary, castration cells; 1.0γ of stilbestrol by vagina, 112 days; ×430.

There was such a wide variety in the observed effects in these experiments it is difficult to say whether any of the doses were able to maintain the uterus in a continuous estrus. It was not uncommon to find one horn of a uterus greatly distended while the other was small and not distended.

In the first series we found great variability of response. There was no relationship between dosage and duration of treatment and the effect, with the possible exception of the time factor in the production of metaplasia. Like Korenchevsky (4) we found that injections of either of the two estrogens was followed by the appearance of metaplasia in the epithelium of the uterus in some animals. The usual columnar epithelium was replaced by the squamous type especially in or near the glands. Such metaplasia occurred on practically all doses of stilbestrol after 52 days of treatment and on the two higher doses of natural estrogens after the same time.

Zondek (6) giving 5,000 M.U. of dimenformon twice weekly to rats for 7 months found partial to total metaplasia of the epithelium with tufts, leucocytic infiltration, varying degrees of distension and in some cases pyometra. He states "I have never seen pictures which one could interpret as malignant tumors or precancerous lesions, if such exist."

In our second series on much higher doses we found, to our surprise, that the changes produced by longer treatment and larger amounts of the estrogens were not different from those in the first series either qualitatively or quantitatively. One exception must be noted. Pyometra was observed in two cases, both accompanied by extensive metaplasia.

Our findings throughout were in remarkable agreement with those recently summarized so well by Zondek (7). "The effect on the epithelium: a) marked variability, focal absence of the epithelium, stratified cylindrical epithelium, tufts and papillae projecting into the lumen, desquamation of whole areas of the mucosa with hydropic cells without visible cell boundaries; b) partial to total metaplasia of the surface epithelium into stratified keratinized squamous epithelum, the uterus having the appearance of a vagina in estrus."

We found no effect of the natural estrogen on the uterus that was not duplicated by stilbestrol and vice versa.

In figure 4 is an example of the type of metaplasia found. Figures 5 and 6 show varying degrees of recovery after cessation of treatment showing that most of these effects are temporary with the possible exception of pyometra and total metaplasia.

Anterior pituitary. Since our sections were cut at the usual thickness for histological work and stained with Mallory's triple stain, cytological distinction of the cell types involving the appearance of the Golgi apparatus, could not be made as described by Wolfe (8), Cleveland (9), Sevringhaus (10) and others. Following Stein (11) in his description of cell types with this stain we have estimated the percentage of different cell types present.

The first striking result noticed was the absence of basophiles in all but a few sections. These, however, together with chromophobes that are distinctly basophilic reappear most consistently in the animals that have a 20-day interval with no injections before autopsy, and those on the lowest dosage of stilbestrol and natural estrogens. Apparently with a cessation of injections, the pituitaries were returning to a normal condition.

With regard to the appearance of dilated sinuses, engorged capillaries, hemorrhage and necrosis, both estrogens seemed to produce these effects, slightly more often in the case of natural estrogens than with stilbestrol. Apparently there is little correlation between dosage and effect.

Zondek (12) starting with rats weighing 55 gm. and giving 5,000 I.U. of estradiol benzoate twice weekly found that after 4 months of treatment the pituitaries of females were grossly normal. He emphasized that the duration of the treatment was of primary importance and the size of the dose of secondary significance. It required at least 7 months treatment before hypophyseal tumors appeared. This agrees essentially with our findings as we did not obtain any great enlargement and no tumors but none of our animals in this series was treated more than three months.

Wolfe (13) using estradiol benzoate found degranulation of the chromophilic cells and marked increase in the chromophobes of the pituitary after 30 to 80 days of daily injections of 200 R.U. Only after more than 6 months of treatment were adenomas formed.

In one animal given 250γ of stilbestrol for 49 days we found a necrotic gland with 80 to 90% eosinophiles. In two other cases both on 500γ of natural estrogens for 28 and 49 days respectively we found small necrotic areas.

In general, then, each of these estrogens produced qualitatively the same changes in the pituitary and evidence indicated these to be of a temporary character.

Thyroid. The thyroids of all the rats in the first series showed varying degrees of destruction of the vesicular walls by invasion of leucocytes, which appeared in the colloid of those that were not intact. In some cases rather large areas occurred in which the acinar structure was completely obliterated and there was left a mass of colloidal material with leucocytes and parts of epithelial cells. In some of these the tissue appeared hyalinized and in others (notably on the 'recovery' rats) these masses had become quite fibrous. Occasionally these areas appeared hemorrhagic.

In the intact vesicles, the epithelium was flat and cuboidal and the colloid eosinophilic indicating a resting gland. Only 2 rats showed the hyperplastic epithelium of an active gland; both had 'rested' 20 days after 52 days of injections, one of 0.5γ of stilbestrol, the other of 2.5γ of natural estrogens.

In the second series the only difference in the picture obtained by higher dosage and longer treatment was fibrosis.

The leucocytic invasion of the vesicular walls has not been previously reported. The significance is not understood. Alexin (14) states that with natural estrogens he obtained histological evidence of heightened functional activity. The thyroids in this series all gave evidence of inactivity. The static condition might be responsible for the infiltration and destruction of the vesicular walls. Grumbrecht (15) in giving estrone, estradiol and stilbestrol daily for 45 days found the thyroid to be in a resting state. Loeser (16) states that in his series of stilbestrol-treated rats the thyroid gave the picture of a resting organ and he attributed this to suppression of the thyrotropic hormone of the anterior pituitary gland. Such changes appear after only 8 to 10 days of treatment. In a more recent paper von Pallos (2) finds both natural and synthetic estrogens produce in the thyroid the histological picture of a resting gland.

In figure 7 there is demonstrated a type of hyaline degeneration which appeared after 15 days, administration of both estrogens. Figure 8 shows moderate infiltration and acinar damage. Figure 9 shows the relatively slight damage obtained on a comparatively large dose.

Adrenal. There was little damage to the adrenals in most of the animals in both series. Many were normal in structure in all respects. Slight to moderate degree of hypertrophy was present in most cases.

On the shortest time of treatment only those on 50γ daily doses of stilbestrol showed any change. With the lower doses over more prolonged time in the first series the effect observed was greatest. Here again apparently the duration of treatment is of greater significance than the total dose of either estrogen.

In the second series swelling and vacuolization of the zona fasciculata was a fairly constant finding, there being little difference in the degree with varying dosage and time. The significance if any is open to conjecture. Korenchevsky (17) found only hypertrophy of the fasciculata in estrone-injected intact male rats which they claimed is found also in uninjected castrate males.

In the second series we have noted necrosis in the glomerulosa and reticularis zones. This appeared in the form of a breaking down of cell structure, leaving the cytoplasm a homogeneous mass with pyknotic nuclei scattered throughout. This condition was strictly limited to focal areas comprising only a small portion of the organ so it probably did not constitute a serious physical upset for the animal nor create any marked hormonal deficiency.

No evidence of brown degeneration of the adrenals as described by Cramer (18) was observed in any of our animals in either series.

Recently von Haam (19) reported marked hyperplasia of the adrenal glands of rats treated with 1 mg. doses of stilbestrol with hyperemia and hemorrhage in the cortical zone. Mazer (20) reports normal adrenals in rats receiving from 75 to 150γ of stilbestrol daily for 42 to 48 days and necrosis of the medulla in rats receiving 1 mg. daily for 45 days.

Liver. Examination of the histology of the liver in rats taken from the first series did not indicate any serious pathological condition. Some showed a slight inflammatory reaction about the blood vessels while others showed a thinning of the cytoplasm of the liver cells rather than a true vacuolization. A more common finding was a blurring of the cell outlines which made the relation of the cells and sinusoids difficult to distinguish. There was no hemorrhage and no necrosis. In a few cases the cell cords were separated by distended biliary sinusoids.

The blurring of the cell outlines mentioned above occurred in rats that had had a rest period of 20 days after 52 days of injections and appeared in those with the lower doses of both estrogens. This occurred in those receiving the three lowest doses of stilbestrol and all (except the lowest) doses of natural estrogens for the shortest time.

In the second series the greatest damage shown was in rats receiving the largest dose of Amniotin for 90 days. All showed recovery after 23 days of rest from injections.

The question of liver damage following the administration of stilbestrol to various species of animals and to humans has been a very controversial subject ever since stilbestrol has been available for experimentation. Grumbrecht and Loeser (15, 16, 21, 22, 23) have reported their studies on the effect of stilbestrol on rats and other animals in which they have jointly or singly emphasized the production of liver damage. Their lowest dose was 250γ of stilbestrol (500 times the physiologic or estrogenic dose). They found (21) degenerative changes after 21 days of treatment but after 90 days on the same dose the livers were completely healed. They assume this to be an adaption phenomenon and claim the time required is dependent upon the daily dose. Even with 1 mg. daily dose at the end of 190 days of treatment both healing and fresh degenerative changes were found. Hackmeister (24) reported liver degeneration in guinea pigs on 1 mg. daily doses. Arnold (25) had one dog on a 5 mg. daily dose of stilbestrol which showed fatty degeneration of the liver. Selye (26) giving equal weights to estrone and stilbestrol, 1 mg. daily, to mice found liver damage resulted from both but more so from stilbestrol. No account was taken of the greater physiologic or estrogenic activity of stilbestrol which might account for some of the quantitative differences. In his experiments von Haam (19) found in his surviving animals a slight cellular anaplasia with some fatty degeneration in one case. Mazer (20) reported a cloudy swelling after 42 days of treatment with 75γ daily in rats and congestion. The dosage required in both rats and rabbits to produce damage is 0.5γ per gm. of

body weight. MacBryde (27) was not able to find any evidence of hepatic impairment from the liver function studies which he made. Freed (28) states that "relatively large doses of stilbestrol in rats and a dog did not give rise to gross or cellular pathologic conditions of liver or kidneys or to liver damage as judged by various criteria of hepatic function. . . . It must be concluded that in our experience stilbestrol is not significantly toxic to the liver."

The trend of opinion in regard to liver damage from the use of stilbestrol has apparently changed from the definitely pessimistic view that it was extremely injurious, to the present idea that it is relatively harmless. We found no evidence in our experimental animals of any serious damage to the liver even with 500 times the physiologic dose.

Examination of the pancreas in these rats did not show any deviation in histologic structure from that of the normal controls. No evidence of any pathology was found in the kidneys.

CASTRATE FEMALE RATS

Procedure. Castrate female rats weighing 150–200 gm. were employed. The completeness of ovariectomy was checked by the occurrence of only diestrous vaginal smears over a period of two weeks. Further confirmation of this was obtained at autopsy.

The experimental animals were divided into four groups. Daily injections of 0.5, 1.0, 5.0 and 50γ of stilbestrol were given in oil solution. Vaginal smears were taken daily. Five rats were placed in each cage and four cages were given the same dosage. At the end of 17, 29 and 52 days of treatment one group of animals on each dose level was autopsied. Following 20 days without treatment after 52 days of injections the remaining group on each dosage was autopsied. The pituitary, thyroid, adrenal, pancreas, liver and uterus were removed at autopsy and fixed in Bouin's solution. Sections were cut, stained and examined. Tissue from at least 2 rats in each cage of 5 was studied. A group of untreated castrates served as controls.

RESULTS

In contrast to the vaginal smears obtained from the intact rats under continuous stilbestrol treatment the castrate animal exhibited continuous vaginal cornification throughout the entire experimental period on all the dosages employed. All animals remained in good physical condition without any deaths and very little if any loss of hair.

Uterus. In the control group the uterus was atrophied, small in cross section with a slit-like lumen. The epithelium was flat, cuboidal and only slightly differentiated from the underlying tissue. Glands were present and well marked from the surrounding edematous tissue of the relatively thick submucosa.

The uteri of the injected rats showed a very similar picture to that found in the injected intact female. The characteristic uterine structure found in all the various stages of the estrous cycle of the normal untreated rat were present in the uterus of this series of animals. There was little relationship between dosage and the effect on the uterine structure.

Metaplasia was observed in only one animal and this rat had received the lower dose (0.5γ) for 52 days.

One uterus showed pyometra, the submucosa was full of leucocytes and pus cells. The mucosa had disappeared. This rat had been treated with the largest dose (50γ) for the shortest time (17 days).

Figure 10 shows the degree of metaplasia found in the one animal in which this occurred in this series. Figure 11 shows a uterus with cuboidal epithelium following

17 days' treatment with ten times the estrogenic dose daily. Figure 12 shows a secreting epithelium following the treatment with 0.5γ of stilbestrol for 17 days.

Anterior pituitary. The untreated castrate female rat exhibits in its anterior pituitary large 'castration' cells which Sevringhaus (10) claims to be basophiles, enlarged and degranulated. In the controls were found 'castration' cells, numerous small brightly stained acidophiles, no small basophiles and few chromophobes.

In the treated animals the castration cells and basophiles were absent as long as injections were continued. After a 20-day rest period following 52 days of injection the gland showed evidence of recovery. Basophilic cells again appeared and in the case of those rats on the lowest dosage in some cases 'castration' cells were found.

Higher dosages seemed to produce more eosinophilic cells with the consequent reduction in the percentage of chromophobes. No interpretation of this is made.

Thyroid. No thyroid gland examined was completely normal even in those animals receiving the smallest dose for the shortest period of time. In this respect the animals in this series presented the same picture as was found in the intact animals injected with the same dosages of stilbestrol. Leucocytic invasion destroyed the acinar walls allowing the colloid to escape into the intervesicular spaces. There was no direct quantitative relationship between the dosage and degree of destruction of the acinar walls. In spite of these thyroid changes the clinical condition of the rats remained very satisfactory.

Three sections have been chosen to depict the maximum degree of destruction, the minimum and the mean between these two degrees. Figure 13 shows the heavy infiltration following only 0.5γ of stilbestrol injected daily for 29 days. Fibrosis is present with acinar structure faintly outlined. Figure 14 shows about 50% destruction of acinar walls. Figure 15 gives the minimum degree of infiltration seen in a rat receiving 5γ of stilbestrol for 17 days.

Adrenal. Considered as a whole the adrenal glands histologically showed very little effect of the injections. The hypertrophy was slight. In untreated animals processes of destruction were noted in some cases in the reticularis. Old cells were 'phaged' by leucocytes and in some cases vacuolation was shown in the cytoplasm of the cells of other zones.

In the injected animals there were no areas of heavy leucoytic infiltration; only a few showed any degeneration of cells or collection of colloid between the cells or distension of blood capillaries or sinuses.

A few exceptions must be mentioned. In two rats receiving 0.5γ of stilbestrol for 52 days there was hemorrhage in the reticularis, some vacuolated cells in the fasciculata and giant cells in the medulla.

In one instance on a dose of 50γ for 52 days there were observed some small areas of degeneration in the cortex. In one rat on 50γ for 52 days edema was present.

Liver. The livers of rats receiving stilbestrol for 17 and 29 days showed so little damage that they might be considered normal. After 52 days of treatment the biliary sinusoids were dilated in some cases with bile compressing the hepatic cells into cords of cells.

After the 20-day rest period following 52 days of treatment effects were found which seemed more serious than any observed while stilbestrol was being administered. In some cases there was blurring of cell outlines, in others a moderate degree of leucocytic infiltration and hemorrhage and in others small necrotic areas. In one case a deposit of greenish yellow granules appeared in the necrotic tissue and in the Kupffer cells.

Figure 16 represents the maximum degree of liver damage which occurred in a rat receiving 50γ of stilbestrol for 52 days followed by a resting period of 20 days. Figure

17 shows moderate damage after the recovery period following 1γ of stilbestrol daily. Figure 18 shows very slight damage in the liver of a rat killed at the end of 29 days of treatment with 5γ of stilbestrol.

In connection with the question of liver damage Huf (4) has made an interesting report of his findings following the administration of several ranges of doses of stilbestrol. He was unable to demonstrate pathological changes after injecting estradiol benzoate, stilbestrol and stilbestrol dipropionate. In his extensive control series of animals receiving no treatment or injections of sesame oil he found all the various types of changes which were observed in his injected animals and claims that the "so-called" toxic effects on the liver of rats reported by Grumbrecht and Loeser (15, 16, 21, 22, 23) were due to their not having control animals from which to observe the variations found in untreated animals.

Certainly in this series there was no evidence of real damage to the livers of animals treated.

INTRAVAGINAL AND PERCUTANEOUS ADMINISTRATION OF ESTROGENS TO CASTRATE FEMALE RATS

The natural estrogens are known to be effective when administered in an ointment applied percutaneously or when given in different vehicles intravaginally. Clinically the use of estrogenic suppositories intravaginally has been effective in treating both senile vaginitis and gonorrheal vulvovaginitis. In this way the desired local effect can be obtained without general systemic effects. Stilbestrol was found to be very active when given vaginally to castrate female rats, at least in the production of complete vaginal cornification. It required only approximately 1/6 as much stilbestrol in oil solution applied intravaginally as it did subcutaneously to produce a completely cornified smear. Freud (30) reported that stilbestrol was 12 times as active by the vaginal route compared with subcutaneous injections. A series of castrate female rats were given a daily dose of 0.1γ and 1.0γ of stilbestrol in 0.1 cc. of corn oil intravaginally. The object of this experiment was to determine whether the estrogenic dose or 10 times that amount would produce general systemic effects even with prolonged treatment.

Palmer (31) reported the estrogenic dose of stilbestrol when given by inunction to castrate mice was 1.0γ. Leighty (32) found in spayed mice the percutaneous estrogenic dose was 0.35γ. Because of the clinical interest in the percutaneous administration of estrogens in certain syndroms it was considered desirable to determine the effect of stilbestrol when administered in large doses in this way. Two menstruums were employed, one a vanishing cream base with stilbestrol and the other a corn oil solution of the substance. In both cases 500γ were given daily in 0.1 cc. of oil or ointment. This was rubbed into shaved areas on the upper part of the abdomen. Daily vaginal smears were taken. Throughout the duration of treatment there were completely cornified smears in all animals in this group.

At the end of 35, 112 and 190 days of treatment animals from each group were autopsied. After a rest period of 56 days following 190 days of treatment other rats from each group were autopsied to determine any changes. As usual in these experiments we removed the pituitary, thyroid, adrenal, pancreas, liver and kidney which were fixed in Bouin's solution and prepared for histological examination.

RESULTS

Anterior pituitary. The rats receiving stilbestrol by the intravaginal route are considered separately from those receiving it percutaneously.

While 0.1γ is approximately twice the dose of stilbestrol which will produce com-

plete vaginal cornification in 75% of castrate females and 1.0γ is approximately 20 times that dose examination of the pituitaries showed that at either dose level the systemic effect was very small if any. Castration cells were present in both these groups with the exception of those in the shortest treatment period. A few basophiles were present in most of the sections examined. Eosinophiles were in all but never in such large amounts as were characteristic of high dosages reported in our previous series of castrates and intact females. With two exceptions there were no pathological conditions found. A slightly hemorrhagic condition was present in rats treated intravaginally with 1.0γ of stilbestrol daily for 112 days.

In the two groups treated percutaneously with 500γ daily in either an ointment base or as an oil solution the systemic effects were present. No castration cells were found in any of the sections. With one exception no basophiles were present. There was no difference between the two groups either qualitative or quantitative showing no advantage for either menstruum.

The essentially normal castrate type of pituitary is shown (fig. 19) in a rat receiving 1.0γ intravaginally for 112 days.

Uterus. Here again the vaginal administration proved to have little effect on the uterus. At no time during treatment was there any distension of the uterus which remained either small or of moderate size. Metaplasia was not found except in one rat which received 1.0γ for 190 days but in this case pyometra was also present. In one case after the recovery period there was some distension and squamous epithelium in the mucosa.

With percutaneous administration the same general picture was found as followed the subcutaneous injection of stilbestrol. Distension of the uterus was found in all cases in the ointment-treated rats and only after 190 days in the oil-treated ones. Cystic hyperplasia, metaplasia and pyometra were observed in some instances. Frequently the two horns of the uterus had entirely different structures. One side might be lined with the usual columnar epithelium and the other with abnormal squamous epithelium. Sometimes the metaplasia was strictly localized in small discrete areas surrounded by normal columnar epithelium.

Thyroid. In both cases whether the stilbestrol were given intravaginally or percutaneously there were considerable changes in the thyroid. We found this with the smaller doses when given subcutaneously. In our experiments changes in the thyroid were found in our controls and the significance must be discounted. In the case of the vaginally treated rats little or no effect was observed in the histological picture of either the anterior pituitary gland or the uterus, but changes occurred in the thyroid similar to those in the percutaneously treated. Probably the thyroid effect is not due to stilbestrol.

Adrenal. The vacuolization in the fasciculata was due to removal of lipoid from cells during the preparation of the section. The instances in which hemorrhage was noted was only a dilation of the capillaries showing an increased number of red cells in the section. The areas of necrosis were extremely small and surrounded by normal tissue. Irrespective of dose or route of administration the adrenal gland was essentially normal.

Liver and kidneys. There was a marked similarity in all sections examined at all dose levels and time intervals. Vacuolization was very slight throughout. There were no hemorrhagic areas anywhere in any sections. A few areas of necrosis were observed but these were very small and of no significance.

The kidneys of these rats were found on histological examination to be essentially normal. The glomeruli of all were normal in every respect. Congestion as evidenced by dilated capillaries was found in some.

SUMMARY

In the intact female rat parallel changes were found in the ovaries, pituitaries, thyroids and adrenals when either Amniotin or stilbestrol was given. Hyperplasia of the pituitary and adrenals was observed on the higher dose levels of either estrogen. No effect was found in the pancreas. No evidence of serious liver damage was found.

In castrate female rats changes comparable to those found in the normal female rats were observed.

Stilbestrol was effective when given intravaginally and it was possible to produce local effects without much evidence of general systemic effects.

Percutaneously, stilbestrol was active either in oil solution or in an ointment. All the effects observed after the subcutaneous injections of stilbestrol were noted after percutaneous application.

There was no evidence of real liver damage in any of these animals irrespective of dosage or duration of treatment. Similar changes were found in control and oil-injected animals.

Pituitary adenomas were never observed but the longest treatment was only 190 days.

Brown degeneration of the adrenals was not found. Little evidence of serious disturbance or damage to the adrenals was presented.

The kidneys, while not examined in all cases, showed no damage.

The pancreas exhibited no change whatsoever in those animals studied.

There were no differences observed in the reaction of the animals to stilbestrol and to the natural estrogens.

REFERENCES

1. Dodds, E. C., W. Lawson and R. L. Noble: *Lancet* 1: 1389. 1938.
2. von Pallos, K.: *Arch. f. Gynak.* 170: 355. 1940.
3. Freud, J., and A. Vedder: *Acta Neerl. Morph* 11: 71. 1938.
4. Korenchevsky, V., and K. Hall: *J. Path. & Bact.* 50: 295. 1940.
5. Long, J. A., and H. M. Evans: Memoirs of Univ. of Calif. Vol. 6, 1922.
6. Zondek, B.: *Am. J. Obst. & Gyn.* 33: 979. 1937.
7. Zondek, B.: Genital Functions and Their Hormonal Regulation, Baltimore, 1941.
8. Wolfe, J. M.: *Am. J. Physiol.* 115: 665. 1936.
9. Cleveland, R.: *Anat. Rec.* 51: 409. 1932.
10. Sevringhaus, A. E.: *Anat. Rec.* 57: 149. 1933.
11. Stein, S. I.: *Endocrinology* 17: 187. 1933.
12. Zondek, B.: *Am. J. Cancer* 33: 555. 1938.
13. Wolfe, J. M., and A. W. Wright: *Endocrinology* 23: 200. 1938.
14. Alexin, M.: *Arch. f. Gynak.* 169: 432. 1939.
15. Grumbrecht, P., and A. Loeser: *Arch. Exp. Path. u Pharm.* 193: 34. 1939.
16. Loeser, A.: *Zeit. f. d. ges. Exp. Med.* 105: 430. 1939.
17. Korenchevsky, V., and N. Dennison: *J. Path. & Bact.* 41: 323. 1936.
18. Cramer, W., and E. S. Horning: *J. Path. & Bact.* 44: 633. 1937.
19. von Haam, E., M. A. Hammel, T. E. Rardin and R. H. Schoene: *Endocrinology* 28: 263. 1941.
20. Mazer, C., S. L. Israel and E. Ravets: *J.A.M.A.* 116: 657. 1941.
21. Grumbrecht, P. and A. Loeser: *Arch. f. expt. Path. u Pharm.* 195: 445. 1940.
22. Loeser, A.: *Klin. Woch.* 18: 346. 1939.
23. Loeser, A.: *Klin. Woch.* 18: 1195. 1939.
24. Hackmeister, W.: *Zentralbl. Gynak.* 63: 2657. 1939.
25. Arnold, O., and H. Hamperl: *Arch. Exptl. Path. u. Pharm.* 194: 121. 1939.
26. Selye, H.: *Can. M. A. J.* 41: 48. 1939.
27. MacBryde, C. M., H. Freedman, E. Loeffel and D. Castrodale: *J.A.M.A.* 115: 440. 1940.
28. Freed, S. C., E. E. Rosenbaum and S. Soskin: *J.A.M.A.* 115: 2264. 1940.
29. Huf, E.: *Klin. Woch.* 19: 915. 1940.
30. Freud, J.: *Acta brevia Neerl.* 9: 11. 1939.
31. Palmer, A.: *Lancet* 1: 1933. 1939.
32. Leighty, J. A., H. J. Wick and B. E. Jeffries: *Endocrinology* 25: 597. 1939.

STUDIES ON STILBESTROL

II. THE EFFECT OF MASSIVE DOSES ON NORMAL IMMATURE FEMALE RATS

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IN THE PREVIOUS PAPER (1) in this series we were unable to find toxic effects of stilbestrol in doses 500 times the physiologic dose. Accordingly we decided to determine the effect of much larger doses over a long period of treatment. Young immature female rats weighing 55 to 65 gm. at the start of treatment were used. In the first group designated as *series A* injections of 1.25 mg. of stilbestrol were given three times weekly. A second group, *series B*, were given 2.5 mg. of stilbestrol three times weekly. A third group, *series C*, served as controls. The animals were weighed at the time injections were given and at autopsy. The body length from tip of nose to tip of tail and tail length were measured at the same times. Very little difficulty was encountered in making these measurements as the young rats quickly became conditioned to the handling and no real restraint was necessary. Vaginal smears were taken daily. At the end of 28, 60, 120 and 150 days of treatment animals from each series were autopsied. The pituitary, thyroid, adrenals, ovaries, uterus, vagina, liver and kidneys were removed, carefully dissected from extraneous tissue and weighed. They were then fixed in Bouin's solution. Sections were cut, stained and examined.

RESULTS

Effects on body growth. It can readily be seen from figure 1 that the administration of stilbestrol caused a marked slowing of body growth but did not altogether prevent such growth. There was no direct quantitative relationship between the size of the dose and the degree of dwarfing. At the end of 150 days of treatment the rats receiving the higher dose of stilbestrol were heavier than the others. In fact, throughout the experiment this same condition prevailed. The effects of the natural estrogens upon body weight, body and tail length were first described by Zondek (2). He has recently (3) summarized his work which has appeared in various sources since that time. In these experiments the reduction in body weight and size was not as great as Zondek has found. Noble (4) reported similar dwarfing following the implantation of crystals of stilbestrol and even greater effects from other synthetic estrogenic substances.

From table 1 it can be seen that the retardation of growth is a general dwarfing. The body length and tail length are proportional to the body weight in all cases and no abnormalities of growth or development were present.

Effects on weight of endocrine glands and some organs (table 2). Because of the great individual variation in the weight of the various glands too much importance must not be attached to differences between the weights for the control and the injected animals. The number of animals in each group was small. The figures presented

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are typical of the general result. These are intended to show trends if any and no significance is attached to minor variations.

Zondek (3) has reported much greater differences in the weights of these glands and liver and kidneys than we found. Noble (4) has reported similar results following the administration of stilbestrol and other synthetic estrogens.

There was a definite increase in weight of the pituitary gland with uniformly greater weights in those rats receiving the higher dose. The difference was slight,

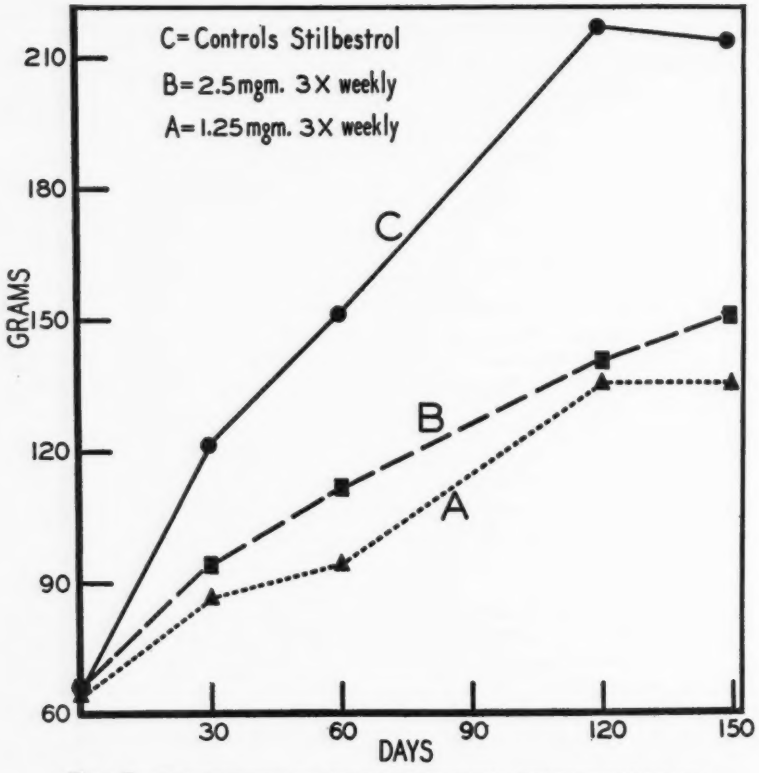

Fig. 1. Effect of stilbestrol on body growth in normal immature female rats.

however, with the exception of the first group under treatment for 28 days. As the duration of treatment increased the differences became smaller. In no case was there anything like the increase reported by Zondek (3).

The thyroid exhibited some increase in size which started after 60 days of treatment and remained throughout the course of the experiment with, however, a trend to diminish as treatment was prolonged. Higher dosages produced heavier thyroids throughout. Freudenberger and Clausen (5) have reported a decrease in the weight of theelin-treated young female rats but the duration and dosage of their experiments were very small compared with those of this report.

The ovarian weight showed considerable variation but in general the treated rats had ovaries smaller than the controls. There was no relationship between dosage and

effect. The decrease in weight was relatively small in consideration of the enormous dosage employed.

The uterus displayed wide variations such as we reported (1) for older rats treated with smaller doses of stilbestrol. Freudenberger and Clausen (5) reported a decrease in weight whereas these experiments showed a trend for the uterine weights of treated animals to be greater than of the controls.

The adrenals showed considerable variation from one animal to another in the

TABLE 1. EFFECT OF MASSIVE DOSES OF STILBESTROL ON GROWTH OF RATS

Treatment period	Total dose	Series	Body length		Tail length	
			Start	Autopsy	Start	Autopsy
days	*mg.*		*cm.*	*cm.*	*cm.*	*cm.*
28	15	A	9.50	11.00	4.37	5.00
		A	9.75	11.37	4.63	5.125
28	30	B	9.50	11.75	4.75	5.37
		B	9.87	12.00	4.62	5.25
28	0	C	9.50	13.25	4.50	6.12
		C	9.75	12.75	4.75	6.25
60	32.5	A	9.25	12.25	4.50	5.75
		A	9.50	11.75	4.75	5.37
60	65.0	B	9.00	12.00	4.50	5.50
		B	9.50	11.87	4.50	5.37
60	0.0	C	9.50	13.87	4.75	6.62
		C	9.00	13.62	4.50	6.25
120	65	A	10.00	12.87	4.87	5.87
120	130	B	9.75	12.87	5.00	6.37
120	0	C	9.75	15.37	4.75	7.12
150	81.25	A	9.25	12.25	4.25	5.50
150	162.50	B	9.25	12.37	4.50	5.62
		B	9.37	12.87	4.50	5.37
150	0.00	C	9.25	14.87	4.50	6.50
		C	10.00	15.50	5.00	7.50
		C	9.25	15.00	4.50	6.62

same group but there was very little difference between the weights of these glands in either treated or control groups. Zondek (3) reported a decrease in weight of the adrenals after treatment with natural estrogens for periods of 20 to 24 weeks. Freudenberger and Clausen (5) reported the difference in his series to be insignificant.

The kidneys of both injected and control rats were of approximately the same weight at the time of autopsy in each series. Zondek (3) found a marked decrease in kidney weight in his series of rats treated with estradiol benzoate. Freudenberger and Clausen (5) found very slight differences but the kidneys weighed less in the treated than in the control rats.

There was no difference in the weights of the livers of the treated and untreated animals. Freudenberger and Clausen (5) found no significant difference. Zondek (3) found the injected rats had livers weighing 21% less than those in the controls.

The pituitary and thyroid showed an increase in weight of the injected rats over the controls. The ovaries exhibited some atrophy and reduction in weight in the

treated animals. The adrenals, liver and kidneys showed no significant difference in this series whether treated or not.

Histological Studies

Ovary. Ovarian activity was decreased and the size of the ovaries decreased by the injection of either 1.25 or 2.5 mg. three times weekly. None of the injected animals

TABLE 2. EFFECT OF MASSIVE DOSES OF STILBESTROL ON WEIGHT OF GLANDS AND ORGANS

Period	Total dose	Series	Pituitary	Thyroid	Ovaries	Uterus and Tubes	Vagina	Adrenals	Kidney	Liver
days	*mg.*		*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*gm.*
28	15.0	A	8.0	8.0	23.5	272	84	22	459[2]	5.98
28	30.0	B	13.5	7.5	10.0[1]	230	79	18	480	5.64
		B	17.0	8.5	25.0	275	128	19[2]	476[2]	5.56
28	0.0	C	8.0	8.5	24.5	232	84	19[2]	514[2]	5.89
		C	8.5	11.5	23	244	135	23[1]	484[1]	4.55
60	32.5	A	12.0	4?	14.5[2]	251	92	30	1024	6.50
60	65.0	B	14.0	16.0	27.0	170	72	62	918	6.05
		B	17.0	17.0	—	—	—	50	943	5.80
60	0.0	C	10.0	12.0	43.5	272	95	44	1189	6.66
		C	10.0	11.0	24.0	334	131	53	985	6.14
120	65.0	A	10.6	10.0	49.0	341	130	44	1021	6.95
120	130.0	B	13.0	13.5	37.2	306	129	56	1146	8.30
120	0.0	C	9.8	9.2	58.0	351	187	51	1396	7.39
150	81.25	A	12.4	11.0	103.6[3]	399	154	40	1397	8.24
150	162.5	B	13.0	14.0	42.0	221	199	43	1225	7.35
		B	16.0	17.0	30.0	153	306	21[1]	1122	6.90
150	0.0	C	8.7	15.6	28.2	249	170	39	1122	6.86
		C	10.0	14.0	55.2	198	105	52	1194	8.44
		C	6.5	11.0	65.6	408	173	43	1362	6.76

[1] Left.
[2] Right.
[3] Foreign growth unidentified; not classified.

had any corpora lutea in their ovaries whereas all of the controls did. Varying degrees of atrophy were found. The connective tissue had become dense and fibrous. Frequently ovaries were seen that contained follicles, in some only a few, and in others, many. A few contained ova. For the most part, however, the follicles were atypical in that they were nearly filled with granulosa cells with little evidence of follicular fluid.

The effects produced seemed in this series to be independent of the size of the dose or the length of treatment. This would be the case if the maximum effect is attained below the lowest dose level and the shortest time interval.

The ovaries in this series were more atypical than those described in our previous report (1). However, there was no malignant growth. Some abnormalities were seen which are never present in the normal ovary. Two pathologists examined these sections independently and they were unable to classify or designate these abnormalities.

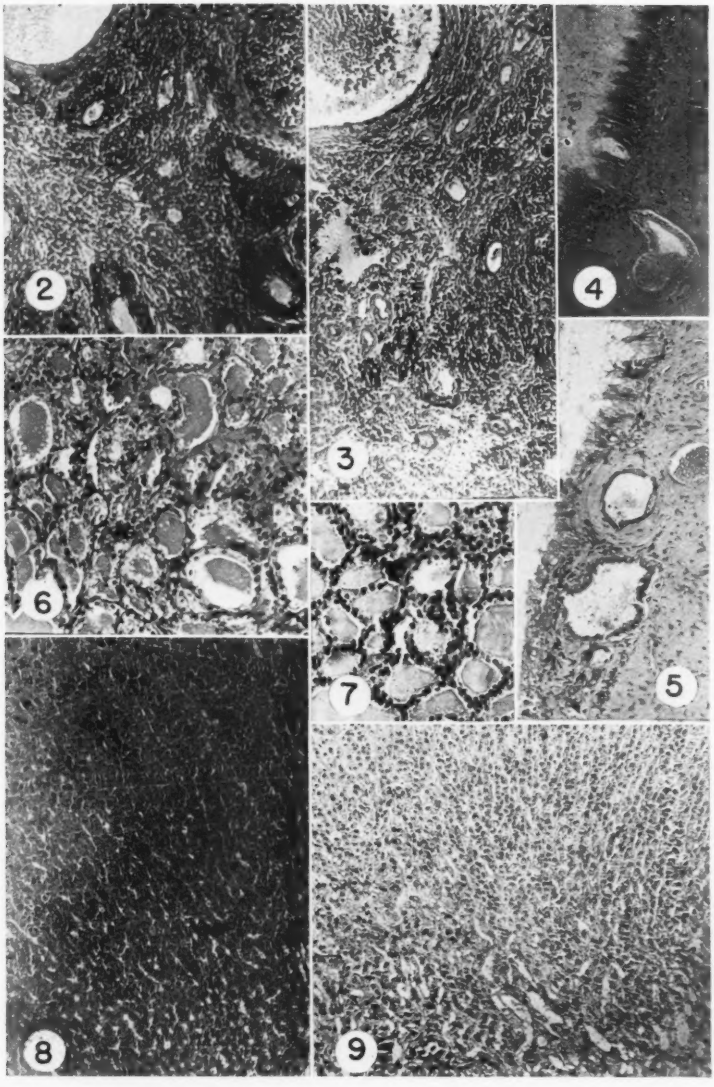

Fig. 2–9. Effects of large doses of stilbestrol on intact female rats. Fig. 2. $C.A_2$. Ovary; no corpora lutea, atrophy; fibrous stroma; follicles degenerate; 65 mg. of stilbestrol, 20 days. ×100. Fig. 3. $C.B_2$. Ovary, no corpora lutea, fibrous stroma, atrophy; 130 mg. of stilbestrol, 120 days. ×100. Fig. 4. $C.B_3$. Uterus; tall columnar epithelium showing some mucification and disintegration; few glands; 162.5 mg. of stilbestrol, 150 days. ×100. Fig. 5. $C.A_2$. Uterus; tall columnar epithelium; disintegrating; glands atrophic; 65 mg. of stilbestrol, 120 days. ×100. Fig. 6. C.A. Thyroid; infiltration of leucocytes and destruction of acinar walls; 15 mg. of stilbestrol, 28 days. ×150. Fig. 7. $C.B_2$. Thyroid; slight infiltration and destruction; epithelium flat; 130 mg. of stilbestrol, 120 days. ×150. Fig. 8. $C.A_1$. Adrenal; slightly hemorrhagic; 32.5 mg. of stilbestrol, 60 days. ×100. Fig. 9. $C.B_3$. Adrenal; normal; 130 mg. of stilbestrol, 120 days. ×100.

Figure 2 shows the ovary of a rat receiving 1.25 mg. of stilbestrol three times weekly for 120 days. Atrophy, absence of corpora lutea, degenerate follicles and dense stroma are all characteristic. In figure 3 we have the resultant ovary following 2.5 mg. of stilbestrol three times weekly for 120 days. In the upper left is shown a follicle nearly filled with granulosa cells. Otherwise the picture is very similar to that obtained on the 1.25 mg. dose.

Uterus. Many interesting contrasts were observed between the effects of these relatively enormous doses and the rather large doses which we reported in our first series.

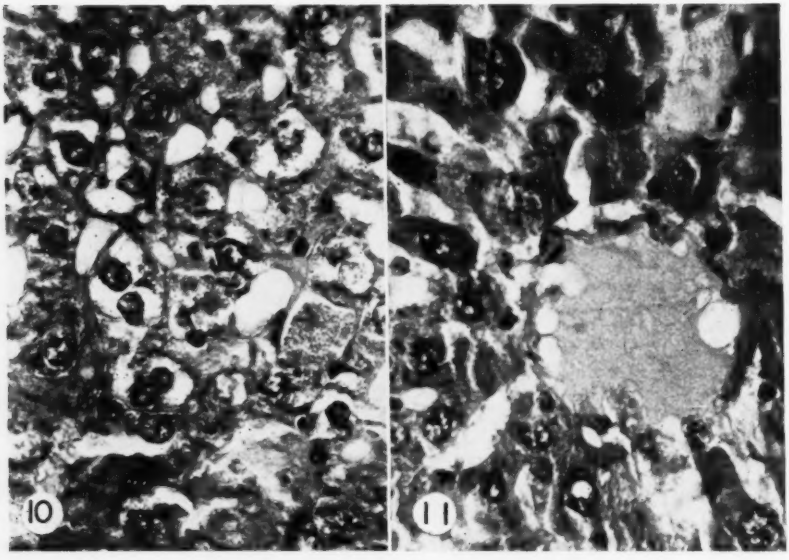

Fig. 10–11. Effect of large doses of stilbestrol on the liver of intact female rats. Fig. 10. $C.B_2$. Some vacuolization; 130 mg. of stilbestrol, 120 days. ×430. Fig. 11. $C.B_2$. Cloudy swelling and edema; 162.5 mg. of stilbestrol, 150 days. ×430.

Metaplasia, found previously only in the higher doses after the longest treatment, was found in the uteri of rats autopsied after the shortest treatment with these relatively large dosages.

Uterine distension which was common on lower dosages was entirely absent. None of the uteri examined were of more than moderate size. The walls were of medium thickness.

The picture obtained is one of a progressive degeneration of the endometrium as the heavy doses of stilbestrol were continued. First metaplasia, then leucocytic invasion of the endometrium and finally pyometra with destruction of most of the regular uterine structures were observed. Only a few specimens showed uterine glands with normal secreting epithelium.

The controls exhibited the usual variations of size, degree of secretory activity and histologic structure which are characteristic of the varying stages of the estrous cycle.

In figure 4, after 162.5 mg. of stilbestrol over 150 days of treatment, are shown the tall columnar epithelium with some mucification and disintegration with few glands present. Following the injection of 65 mg. over a period of 120 days, figure 5 shows the same type of epithelium also disintegrating. More glands are present but they are atrophic.

It may be significant that no tumors or adenomas were found even after prolonged treatment with such large dosages.

Recently Russell (6) and associates have reported some studies on the oral administration of large doses of stilbestrol to rats giving as high as 15 mg. per week. In normal female rats they found endometrial hyperplasia in the uterus and not metaplasia or results comparable to those reported here. This may be due in part to the lesser degree of activity when stilbestrol is given orally.

Neither Grumbrecht and Loeser (7), Loeser (8) nor von Pallos (9) have found any uterine changes beyond metaplasia although they gave 1 mg. of stilbestrol and stilbestrol dipropionate daily either by injection or orally.

Anterior pituitary. The anterior lobe of pituitaries of the injected rats were typical of estrogen-treated animals in that they contained no basophiles. On the larger dosage, 2.5 mg., the eosinophiles were noticeably more numerous than in the other group. In one group after 60 days of treatment with 65 mg. of stilbestrol the percentage of eosinophiles were remarkably high but it was not however great enough to warrant the term 'eosinophilic adenoma.' With longer treatment the number was reduced again. The chromophobes made up the greater part of the cell components of the gland.

Hyperplasia was noted but in no case were evidences of adenoma or tumors found.

Thyroid. The general picture found in the thyroids of these animals was rather puzzling. In both injected and control animals similar kinds and degrees of damage were found. This would indicate that the changes cannot be ascribed to the injection of stilbestrol. Furthermore at the end of 150 days of treatment with either 81.25 or 162.5 mg. of stilbestrol the glands were essentially undamaged and corresponded very favorably if not advantageously to those of the control rats of the same age.

The question arises whether this course of events can be ascribed as Grumbrecht and Loeser (10) have done to an adaptation phenomenon where the animal becomes adjusted to the large doses and the organ or organs regain their normal structure and function.

In figure 6 are shown the maximum changes which were observed in any of the thyroids studied. In this case the rat had received 15 mg. of stilbestrol during a period of 28 days. The degree of recovery shown during the course of treatment is shown in figure 7. Infiltration is slight, destruction of acinar walls small and the epithelium is flat. This compares very well with the thyroid from normal untreated animals.

Adrenal. In spite of the enormous dosage, the adrenals showed very little damage. Beyond some degree of hyperplasia the changes were slight. A small amount of hemorrhage in the cortex was found in some cases.

In figure 8 the hemorrhagic condition is shown. This rat had received 32.5 mg. of stilbestrol over a period of 60 days. In figure 9 is an essentially normal adrenal of a rat which received 130 mg. of stilbestrol during a period of 120 days.

Liver and kidney. Cloudy swelling was found in the livers of most of the injected animals. While this is considered the first stage of necrosis there were only microscopic areas of real necrosis even after 150 days of treatment. Edema may be found

in normal livers under certain conditions of alimentation and its occurrence here probably has no significance.

In one instance a fatty tumor was observed and was probably due to factors other than the stilbestrol.

In no case was there any evidence of marked widespread necrosis, degeneration or fatty infiltration adequate to give any degree of impairment of liver function.

Vacuolization in moderate degree is shown in figure 10 in a rat receiving 130 mg. of stilbestrol over a period of 120 days. The cloudy swelling and edema found in some animals is shown in figure 11. This rat had received 162.5 mg. of stilbestrol during 150 days of treatment.

Kreitmair (11) and Sieckmann giving 10 mg. daily to rats found that their oil-treated controls showed more fatty degeneration of the liver than the stilbestrol-treated animals. They observed some evidence of an increase in weight of the liver following increasing dosages of stilbestrol. They occasionally saw small areas of focal necrosis which they believed were unrelated to the treatment with stilbestrol.

The kidneys of both treated and control rats in our experiments were for the most part normal. The glomeruli of all were undamaged and there was no thickening of the blood vessels. In all animals there was breaking down of the cytoplasm of the cells lining the tubules with debris in the lumen but this was probably due to autolysis before fixation. As it was present in all controls as well and to about the same degree the pathologic significance was reduced to a minimum.

Kreitmair (11) found no abnormalities in the kidneys of the rats given as much as 10 mg. of stilbestrol daily. Russell (6) found cloudy swelling in some of the kidneys but did not attribute this to stilbestrol as it was present in control rats as well.

SUMMARY

The changes in the endocrine glands following treatment with stilbestrol were characteristic of estrogenic treatment. Even in the large doses employed no definite evidence of toxicity of stilbestrol was obtained. The highest dosage on a weight basis corresponded to 97,500 mg. for a 60-kg. woman whereas the average maintenance dose of stilbestrol for a menopausal patient is approximately 1 mg. daily. Consequently there is a wide margin of safety if any comparison can be drawn between the reactions of these two widely divergent species.

REFERENCES

1. Morrell, J. A., and G. W. Hart: *Endocrinology* 29: 796. 1941.
2. Zondek, B.: *Lancet* 2: 842. 1936.
3. Zondek, B.: Genital Functions and Their Hormonal Regulation, Baltimore, Md.
4. Noble, R. L.: *Lancet* 2: 192. 1938.
5. Freudenberger, C. B., and F. W. Clausen: *Anat. Rec.* 68: 133. 1937.
6. Russell, H. K., R. C. Page, C. S. Matthews, E. L. Schwabe and F. E. Emery: *Endocrinology* 28: 897. 1941.
7. Grumbrecht, P., and A. Loeser: *Arch. f. exper. Path. u. Pharmakol.* 193: 34. 1939.
8. Loeser, A.: *Zeit. f. d. ges. Exper. Med.* 105: 430. 1939.
9. von Pallos, K.: *Arch. f. Gynak.* 170: 355. 1940.
10. Grumbrecht, P., and A. Loeser: *Arch. f. exper. Pathol. u. Pharmakol.* 195: 445. 1940.
11. Kreitmair, H., and W. Sieckmann: *Klin. Wochnschr.* 19: 474. 1940.

EFFECT OF LARGE DOSES OF ESTROGENS ON THE BLOOD PICTURE OF DOGS

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ANEMIA IN DOGS CAN BE PRODUCED by the administration of large doses of estrogens, such as estradiol benzoate, estrone (1–6)[2] and diethylstilbestrol (7, 8),[2] that is by substances of varied constitution whose common characteristic is estrogenic activity. Anemia can be produced in both sexes by this means. On the other hand androgenic substances such as testosterone or its propionate do not produce an anemia even after injection for a period of months (6).

METHODS

Healthy, vigorous dogs were used for the experiments. They were kept on a mixed diet which contained all the known vitamins. Before the investigation was commenced they lived for at least one week in their new surroundings. Blood was taken from the ear of fasting dogs and was analyzed immediately without the addition of anticoagulants. Hemoglobin was measured by Sahli's method and the number of blood corpuscles determined in the Thoma-Zeiss chamber. Reticulocytes, stained with cresyl blue, were counted per 1,000 red blood cells. The normal values found for 21 dogs, 16 females and 5 males, were as follows. Average number of red blood cells 6.42 millions; hemoglobin content (Sahli) 97.7% (Sahli 100% equal to 17.4 gm. oxyhemoglobin per 100 cc. blood); reticulocytes 0.46%; thrombocytes 304,000; white blood cells 12,670. For a summary of the normal values for dog's blood reference may be made to Scarborough (9) and Wintrobe, Shumacker and Schmidt (10).

The dogs received daily subcutaneous injections of 5 mg. of the crystalline substance in question dissolved in 1 cc. of olive oil. Three dogs were injected daily with 1 mg. doses. Blood determinations were carried out twice weekly.

RESULTS

The sequence of alterations in the blood. Seven females and one male were injected with 5 mg. of estradiol benzoate daily, 3 females with 5 mg. of stilbestrol daily. Two females were daily treated with 1 mg. of estradiol benzoate and 1 female with 1 mg. of stilbestrol. In order to determine if the toxic action is connected with the presence of ovaries, uterus or vagina, experiments on 2 animals (*dogs* 4 and 5, tables 1 and 3) were carried out in which the uterus and the vagina were removed before the beginning of the experiment. In a third animal (*dog* 3), the ovaries were removed in addition. All animals, normal and castrated females and the male which were injected with 5 mg. daily showed the same degree of anemia. A daily dose of 1 mg. of estradiol benzoate and stilbestrol, respectively, given continuously for 9 months, produced no ill effects.

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[2] Since this paper went to press the papers by Castrodale, Bierbaum, Helwig and MacBryde (22) and by Crafts (23) have been published.

On daily injection of 5 mg. of estrogens a fall occurred in nearly all of the blood elements after a period of 1 to 3 weeks (fig. 1 and table 1). The decrease of the white blood cells was usually preceded by an initial rise. Changes in the individual blood

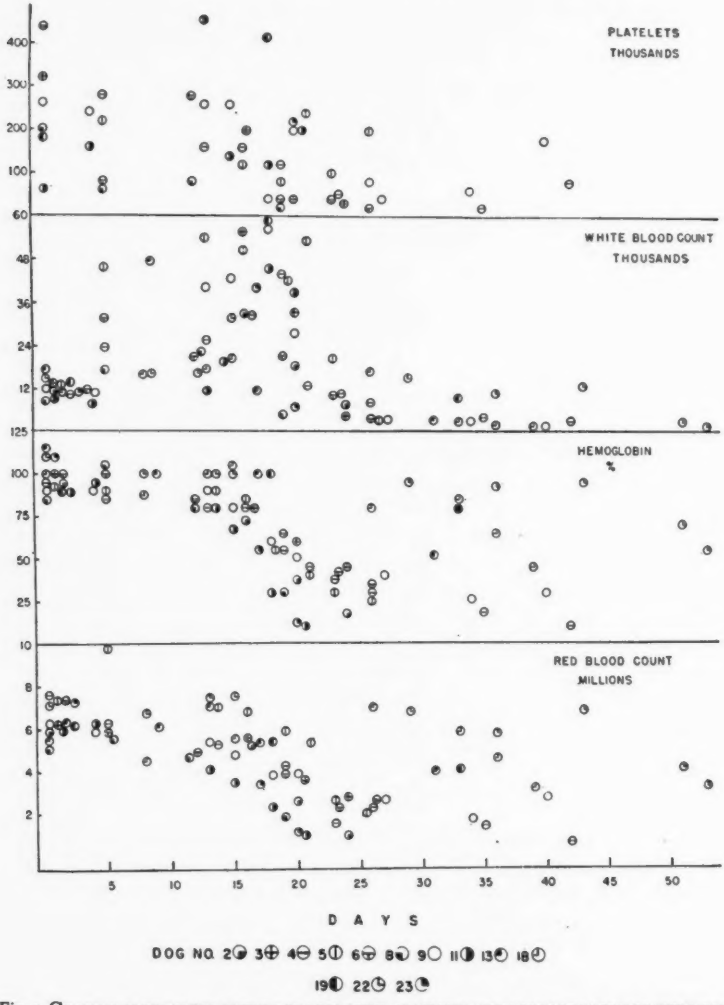

Fig. 1. CHANGES IN THE BLOOD PICTURE OF DOGS AFTER ADMINISTRATION OF LARGE DOSES OF ESTROGENS. Most uniform change observed was a decrease in the number of white blood cells.

elements did not run parallel; a normal number of red cells or even a decrease sometimes occurred concomitantly with a leukocytosis (*dogs 2, 3, 4, 5, 6, 9, 11, 23*). The disappearance of leukocytosis and the diminution in the white blood cells especially the granulocytes was a serious prognostic sign, usually accompanied by a fall in the other blood elements. The degree of the diminution in the individual blood elements

TABLE 1.—EFFECT OF LARGE DOSES OF ESTROGENS ON BLOOD PICTURE OF DOGS

Dog no., sex	Time on treatment days	W.B.C. per c. mm., thousands	R.B.C. per c. mm. millions	Hemoglobin Sahli %	Platelets, thousands	Reticulocytes %	Weight, kg.	Treatment and remarks
				Estradiol benzoate, diethylstilbestrol in normal dogs				
6	0	12.2	7.38	100	440		17.0	Estradiol benzoate 5 mg. daily
♀	5	31.6	5.94	100	80		16.5	from 3/16/38.
	12	20.8	4.92	85	280		16.9	
	19	21.2	3.92	65	40		17.4	
	23	10.0	2.30	40	40		18.2	Died on 33rd day after 32 injec-
	26	3.4	2.44	35	20		17.2	tions.
8	0	8.4	5.10	85	200		9.0	Estradiol benzoate 5 mg. daily
♀	5	17.2	5.60	104	60		9.3	from 3/16/38.
	12	21.9	4.84	81	80		9.0	
	19	4.6	1.86	30	20		9.3	Prolonged bleeding time, vaginal bleeding; died on 27th day after 26 injections.
2	0	11.8	6.10	95	280		7.6	Estradiol benzoate 5 mg.
♀	16	33.0	5.32	72	240		8.3	daily from 1/19/38.
	20	18.6	2.60	38	140		8.8	Died on 25th day after
	24	7.6	0.96	17	500		7.6	25 injections.
18	0	11.6	5.53	100		0.4		Diethylstilbestrol 5 mg. daily
♀	8	16.0	4.54	87		1.2		from 9/15/38.
	13	17.6	7.10	100		2.8		
	15	20.4	5.56	105		0.4		
	26	16.8	7.06	80		4.0		
	33	2.4	5.88	85		0.1		
	36	1.0	4.64	65		0.2		Died on 41st day after 41
	39	0.4	3.18	45	220	0.1		injections.
22	0	14.8	7.15	110		0.1		Diethylstilbestrol 5 mg. daily
♀	8	16.2	6.78	100		—		from 9/15/38.
	13	16.2	7.42	100		0.3		
	15	30.2	7.52	100		1.6		
	29	15.0	6.80	95		4.0		
	36	10.6	5.90	87		1.6		
	43	12.6	6.86	95	260	0.4		No injections after 51st day.
	51	1.2	4.16	70	220	0.8		Died 4 days after 51st injec-
	53	0.6	3.32	55	280	0.0		tion.
13	0	17.2	6.34	115	200	0.4	11.8	Estradiol benzoate 5 mg. daily
♂	9	47.4	6.16	100	260	—	13.5	from 6/26/38. Dose increased
	17	11.6	5.34	100	920	—	14.0	to 10 mg. daily from 33rd day
	31	3.2	3.98	52	—	0.8	14.5	on. Died 41st day after 41 inj.
				Estradiol benzoate in castrate females				
3	0	12.4	6.30	100	320		9.7	Uterus, ovaries and part of
♀	16	55.6	5.60	80	200		9.4	vagina removed before treat-
	20	33.4	3.58	60	40		9.2	ment with estradiol benzoate
	24	4.1	2.77	45	30		9.5	5 mg. daily. Died on 27th day, 2/15/38 after 27 injections.
4	0	10.4	7.62	95	760		9.1	Uterus, vagina removed
♀	5	23.8	6.30	85	280		8.0	3/15/38.
	13	25.6	5.36	80	160		8.0	Estradiol benzoate 5 mg. in-
	16	32.6	—	80	160		7.8	jected daily from 3/16/38.
	19	43.8	4.26	55	120		7.9	
	21	12.6	—	45	—		8.2	
	23	11.0	1.76	37	40		8.4	
	26	7.8	2.26	30	80		8.4	

TABLE 1—*Continued*

Dog no., sex	Time on treatment days	W.B.C. per c. mm., thousands	R.B.C. per c. mm., millions	Hemoglobin Sahli %	Platelets, thousands	Reticulocytes %	Weight, kg.	Treatment and remarks
	35	3.6	1.42	18	20		—	Died on 44th day, after 44 injections.
	42	2.6	0.64	10	80		—	
5	0	13.0	7.44	93	800		12.0	Uterus, vagina removed on 3/15/38.
♀	5	45.6	9.80	90	220		12.0	
	13	54.0	7.04	90	120		—	Estradiol benzoate 5 mg. injected daily from 3/16
	16	50.8	6.80	85	120		12.1	
	19	42.2	5.90	55	80		12.3	
	21	53.2	5.38	40	240		12.6	
	23	20.4	2.64	30	100		12.5	Died on 32nd day, after 31 injections.
	26	3.6	2.14	25	200		12.6	
				Testosterone propionate				
10	0	9.6	6.10	100	400	0.8	10.4	Injected daily with testosterone propionate 10 mg. for 81 days; Mar., Apr., and May 1938.
♂	3	11.6	5.52	87	140	0.1	10.3	
	9	10.4	6.90	88	380	0.1	10.2	
	20	13.2	6.08	95	100	0.3	10.5	
	27	15.0	5.92	95	160	—	10.2	
	39	13.4	5.96	90	100	0.3	10.3	Dog remained in good condition until killed, last day of experiment.
	67	9.2	5.24	100	60	0.8	10.3	
	74	18.0	7.00	105	180	0.8	10.2	
	81	10.2	5.86	95	100	0.2	10.2	
12	0	11.2	5.80	100	300	0.4	12.4	Injected daily with testosterone 5 mg. for 63 days; from 63rd day on dose increased to 10 mg. daily and 20 more injections given. Jan., Feb. and Mar. 1938.
♂	3	16.0	6.10	100	600	0.4	12.5	
	6	11.4	6.30	95	140	0.3	12.5	
	10	11.2	5.60	85	560	0.8	12.5	
	13	7.8	5.16	92	380	0.8	12.4	
	17	7.4	—	90	400	—	12.5	
	20	7.2	6.88	95	520	0.8	—	
	23	10.8	6.95	92	360	—	—	
	27	8.4	6.72	100	340	—	12.6	
	31	11.4	5.52	90	300	0.4	12.3	
	35	7.2	—	105	300	0.1	12.5	
	..38	11.6	6.24	105	400	0.1	12.4	
	55	6.4	6.44	95	380	0.1	—	
	62	4.8	6.16	95	240	—	12.7	
	68	7.6	6.00	95	280	—	12.8	Dog remained in good condition until killed on last day of experiment.
	78	6.0	5.30	95	100	0.1	12.7	
	83	14.0	5.15	90	220	—	12.8	
				Estradiol benzoate and testosterone				
19	0	14.0	6.20	90	180	0.1	10.2	Injected daily with estradiol benzoate 5 mg. and testosterone 10 mg. until 29th day; from 30th until 37th day, dose of estradiol benzoate increased to 10 mg. daily. Treatment was stopped 38th day. Dog died 1 wk. later. June-Aug. 1938.
♂	18	59.4	5.40	100	420	1.6	11.8	
	33	9.4	4.08	80	—	3.2	11.8	
23	0	10.6	7.16	110	—	0.4	9.7	Injected daily for 20 days with estradiol benzoate 5 mg. and testosterone 5 mg.; dog died on 24th day. Oct. 1938.
♂	17	40.2	3.42	55	—	0.4	10.2	
	20	6.6	1.10	12	220	2.8	—	

TABLE 1—*Continued*

Dog no., sex	Time on treatment days	W.B.C. per c. mm., thousands	R.B.C. per c. mm., millions	Hemoglobin Sahli %	Platelets, thousands	Reticulocytes %	Weight, kg.	Treatment and remarks
				Estradiol benzoate and liver extract				
9	0	11.8	6.36	90	260	0.4	14.5	Estradiol benzoate 5 mg. daily
♀	4	9.8	5.94	90	240	0.1	14.7	injected until 20th day.
	13	40.6	5.42	90	260	—	15.6	
	15	42.8	4.80	80	260	—	15.3	Liver extract 4 cc. daily in-
	18	56.4	3.82	60	40	0.4	—	jected from 16th day on; dose
	20	27.6	3.86	51	220	2.0	—	increased to 8 cc. daily from
	27	3.4	2.64	40	40	0.2	—	19th day on. In addition 10 cc.
	34	2.8	1.74	26	60	0.4	—	liver extract given intrave-
	40	2.4	2.68	30	180	0.8	—	nously on 30th day. No injec-
	42	6.8	2.38	30	160	—	—	tions after 54th day. On 61st
	45	5.0	1.88	30	180	—	—	and 62nd day 500 mg. of nico-
	54	3.6	2.08	32	180	3.2	14.5	tinic acid per mouth.
	61	—	3.26	42	160	2.4	14.3	
	62	—	—	62	—	—	—	
	63	6.5	3.82	60	260	2.4	—	
	66	11.0	4.08	62	—	—	14.6	
	70	7.2	4.26	65	200	—	14.8	
	78	6.8	5.00	80	—	—	14.7	
	84	11.2	6.40	95	280	—	14.3	Injections of estradiol benzoate
	113	17.2	4.40	75	360	—	16.0	5 mg. daily started again on
	118	4.2	4.40	58	—	—	16.7	94th day. Animal killed on
	124	2.2	3.16	56	—	—	15.7	126th day. Mar.-July 1938.
11	0	10.4	6.04	90	60	0.4	7.0	Estradiol benzoate 5 mg. daily
♀	4	7.8	6.26	92	160	0.3	7.1	injected until 20th day. Liver
	13	11.6	4.10	80	600	—	7.2	extract 4 cc. daily injected from
	15	20.0	3.48	67	140	—	7.5	16th day on; dose increased to
	18	46.0	2.26	30	120	1.2	—	8 cc. daily from 19th day on.
	20	39.2	1.06	10	200	1.2	—	Died on 21st day. Mar.-Apr. 1938.

at the time of death varied (fig. 1). The values for the platelets at death were usually low, but in some cases they were normal (*dogs 2, 5, 11, 18, 22, 23*). The red blood count in the greater number of cases was below 3 millions at the time of death, but in *dogs 18* and *22* death occurred when the red blood count was above this level. Death during the phase of leukocytosis also occurred, as in *dog 11*. Sometimes an increase in reticulocytes was followed by a second rise in the red blood count (*dog 18*). The fall in the number of red blood cells was initially much more rapid than the fall in the hemoglobin content, so that at first the anemia was hyperchromic; later with more intense anemia, it became normo- or hypochromic. Significantly in later stages there was a prolonged bleeding time. Some dogs bled for an hour or longer from the small puncture which was made in the ear to obtain the blood sample. When the incision was made there was a delay before blood appeared; once started the hemorrhage was difficult to stop.

The initial leukocytosis was accompanied by a relative lymphopenia (table 2), while the immature forms of the polymorphonuclear elements were increased. Marrow obtained by sternal puncture at this time showed an increase in maturing white cell elements. The subsequent decrease of circulating leukocytes particularly affected the granulocytes, so that a relative lymphocytosis occurred. Microscopic examination of the bone marrow at this time usually revealed a decrease in the maturing granulocytes. Finally, only quite isolated granular elements were observed in the

TABLE 2. DIFFERENTIAL BLOOD CELL COUNTS IN DOGS TREATED WITH ANDROGENS AND ESTROGENS

Dog no., sex	No. of days	W.B.C. per c. mm., thousands	Differential count								Remarks
			Myelocytes	Juveniles	Stab	Neutrophiles	Eosinophiles	Basophiles	Lymphocytes	Monocytes	
Testosterone propionate											
10 ♂	0	9.6	—	—	3	50	5	—	39	3	
	9	10.4	—	—	2	55	3	—	37	3	
	20	13.2	—	—	3	58	2	—	35	2	
	39	13.4	—	—	5	57	1	—	32	5	
	67	9.2	—	—	4	62	3	—	28	3	
	74	18.0	—	—	3	65	3	—	23	6	
Testosterone											
12 ♂	0	11.2	—	—	1	77	2	—	18	2	
	35	7.2	—	—	4	61	6	—	26	3	
	55	6.4	—	—	5	62	4	—	24	5	
	68	7.6	—	—	5½	68½	1½	—	22	2½	
	78	6.0	—	—	1	67	5	—	24	3	
Estradiol benzoate											
9 ♀	0	11.8	—	—	1	68	3	—	26	2	
	18	56.4	—	3	6	67	3	—	19	2	
	27	3.4	—	—	3	37	2	1	55	2	
13 ♂	0	17.2	—	—	2	65	6	—	26	1	
	31	3.2	—	—	—	5	—	—	95	—	
Estradiol benzoate and liver extract											
16 ♀	0	10.4	—	—	2	56	10	—	27	5	
	14	33.4	—	—	5	74	6	—	10	5	
17 ♀	0	10.4	—	—	4	58	10	—	26	2	
	14	43.8	—	—	1	68	5	—	21	5	
	21	30.4	—	—	1	89½	3	—	6½	—	
Diethylstilbestrol											
18 ♀	0	11.6	—	—	4	67	5	—	20	4	
	13	17.6	—	2	12	63	7	—	12	4	
	15	20.4	—	6	18	59	3	—	8	6	
	26	16.8	—	1	2	74	8	—	15	—	numerous normoblasts
	33	2.4	—	—	2	7	5	—	85	1	macrocytes
	36	1.0	—	—	—	3	—	—	97	—	
22 ♀	0	14.8	1	2	7	65	7	—	15	3	
	13	16.2	1½	4½	6½	69½	2½	—	12½	3	
	15	30.2	—	1	8	72	2	—	6	11	
	29	15.0	—	6	13	63	2	—	16	—	
	51	1.2	—	5	9	37	—	—	48	1	numerous normoblasts
	53	0.6	—	8	2	8	—	—	82	—	

blood stream. This picture of almost complete absence of granulocytes in the peripheral blood, accompanied by a decrease in the granulocytes and myelocytes in the bone marrow with necrosis of the mucous membranes and skin resembled that described in man as "agranulocytosis" by Schultz (14). It was remarkable that in such cases granulocytopenia occurred quickly and developed within a few days (fig. 1, table 1 and 2). In other dogs, the picture was that of thrombocytopenic purpura.

Clinically the animals appeared quite normal during the early stages of the experiment, even when the anemia was marked. Later, however, in most cases bleeding occurred under the skin and mucous membranes. Finally blood escaped from the vagina and the intestines, appearing with the feces or in the vomited masses. In

the last few days the dogs lost their appetite, became apathetic and showed dyspnea, but as a rule they retained their attentiveness until the end. It is noteworthy that frequently edema occurred around the mouth, similar to the sexual skin alteration around the mouth of the ape (11, 12, 13). In most cases the animals showed edema as the water and weight balance was usually positive from the beginning of the injections. Toward the end of the experiment, loss in weight occurred. Sometimes the dogs lost their hair; in the final stages, healing of wounds was poor.

Autopsy Findings

The edema was noticeable on cutting the skin or muscle. The necrosis mentioned occurred especially on the toes, around the mouth and on the skin of the abdomen. It was also found in the region of the respiratory tract and the alimentary canal. Frequently numerous hemorrhages were observed under the skin and the mucous membrane of the alimentary canal or the urogenital tract. In one case the uterus was filled with blood clots and in another animal the rectum and the bladder were covered with petechiae. Hemorrhages occurred under the pleura, the epi- and endocardium, especially in the neighborhood of the large papillary muscles and in the region of the abdomen under the serosa of the viscera. At microscopic examination hemorrhages were observed in the spleen, adrenals, thymus and lungs. In some cases, however, hemorrhages were entirely absent.

The heart muscle was flaccid and friable. The lungs were relatively rich in fluid and showed edema or hemorrhages and sometimes pneumonic centers were found. According to the severity of the anemia the parenchymatous organs were more or less depleted of blood. Usually the liver was not enlarged and the spleen was often smaller than normal; in both organs hemosiderin was found. The lymphatic tissue in the lymphatic glands, in the thymus and spleen seemed to be unaffected. The color of the sternal bone marrow varied according to the severity of the anemia. The correlation usually found was a paler bone marrow with the more severe grades of anemia. Reference may be made to Arnold, Hamperl, Holtz Junkmann and Marx (2) the results of whose studies on bone marrow were confirmed. At microscopic examination the bone marrow of dogs which died with a low red and white blood count showed few areas of regeneration. The hypophysis weighed 30 to 119 mg. which is considered normal as the weight of the hypophysis in dogs varies widely according to the breed. In the histologic study of the hypophysis of the dog it is difficult to determine the relation in the number of the chromophobic, eosino- and basophilic cells since this varies with different breeds. Therefore it is difficult to make any statement about the preponderance of certain cells. The genital tract showed the characteristic effects of treatment with large doses of estrogens, Hyperplasia of the uterus and swelling of the vulva were particularly marked.

The death of the dogs may be explained by anemia, pneumonia or infections. In cases with granulocytopenia, microorganisms entered into the blood stream; blood preparations initially were free from microorganisms, and later numerous spirilli, bacteria and cocci were observed. Such cases always terminated fatally. A similar observation was made by Gardner and co-workers (5). The direct cause of death may be circulatory failure which may occur in some cases rather suddenly. Thus, for example, dog 23 was relatively lively in his cage and when he was lifted out by hand for a blood test, he actually wagged his tail; when the animal was placed on the floor, however, it was dead. In this, as in other cases, a very flabby heart muscle was found.

Toxic Doses

Individual variations occurred among the animals as regards their sensitivity to the action of large doses of estrogens. Light colored, young animals and inbreds

TABLE 3. EFFECTS OF LARGE DOSES OF ESTROGENS ON DOGS

Dog no., sex	Weight kg.	Daily dose mg.	No. of days of injection	Total amount mg.	Total amount, mg./kg.	Colour of skin	Remarks
			Estrone				
1♀	13.0	5	19	95	7.3	white	Died after 19 injections
			Estradiol benzoate				
2♀	8.0	5	25	225	15.6	black	Died after 25 injections
6♀	17.0	5	32	160	9.4	spotted	Died after 32 injections
8♀	9.0	5	26	130	14.5	spotted	Died after 26 injections
13♂	12.9	5 10	32 9	250	19.4	dark brown	From 33rd day on dose increased to 10 mg. daily, 9 more injections given. Died after 41 injections
15♀	11.0	5 5	14 25	195	17.8	white brown	After 14 injections experiment interrupted for 3 wks., then 25 more injections given. Died 1 day after 39th injection.
18♀	14.5	5	20	100	6.9	brown	Survived experiment; after 10 wks. experiment repeated with diethylstilbestrol. Died after 41 injections.
			Diethylstilbestrol				
	17.5	5	41	205	11.7		
21♀	10.9	5	13 31	220	20.1	black	After 13 injections experiment interrupted for 10 days, started again and 31 injections given; animal killed when severly anaemic.
22♀	16.5	5	51	255	15.5	black	Died 4 days after 51st injection.
			Estradiol benzoate in castrated females				
3♀	9.0	5	27	135	15.0	spotted	Ovaries, uterus and vagina removed at beginning of experiment. Died after 27 injections.
4♀	8.0	5	44	220	27.5	spotted	Uterus and vagina removed at beginning of experiment. Died after 44 injections.
5♀	12.0	5	31	155	12.9	fair	Uterus and vagina removed at beginning of experiment. Died after 31 injections.
			Estradiol benzoate and testosterone				
19♂	11.1	5 10 10	29 8 37	225 370	19.7 33.3	dark brown	Injected daily with estradiol benzoate 5 mg. and testosterone 10 mg. for 29 days; for 8 days, estradiol benzoate increased to 10, mg. daily while testosterone kept same. Died 1 week after the 37th injection.
23♂	10.0	5 5	20 20	100 100	10.0 10.0	white	Very young animal. Died 4 days after 20th injection.

TABLE 3—*Continued*

Dog no., sex	Weight kg.	Daily dose mg.	No. of days of injection	Total amount mg.	Total amount, mg./kg.	Colour of skin	Remarks
			Testosterone propionate and testosterone				
10♂	10.3	10	81	810	78.6	brown	Testosterone propionate. Dog remained in good condition.
12♂	12.5	5 10	63 20	515	41.2	black	Testosterone. Dog remained in good condition.
			Estradiol benzoate and liver extract				
9♀	15.0	5	20	100	6.67	light brown	+ liver extract from 16th day on; survived experiment.
11♀	7.0	5	20	100	14.2	white black	+ liver extract from 16th day on; died on 21st day.
16♀	15.5	5	25	125	8.0	grey	Liver extract injected before and during entire course of estradiol benzoate administration. Died 4 days after last injection.
17♀	11.6	5	25	125	10.77	black	Liver extract injected before and during entire course of estradiol benzoate administration. Survived experiment.

seemed to be more sensitive. Since injections were usually continued until the appearance of severe symptoms, which were rapidly followed by death, the doses listed in table 3 are not necessarily minimal toxic doses, for it must be remembered that the toxic effects may occur some time after injections have ceased, depending on the amount taken. The data on dog 9 (table 1) demonstrate the fact that the anemia continues its course after discontinuing the injections of estrogens. The results given in table 3 show that with estradiol benzoate dogs will remain alive with a total dose of 5 to 7 mg. per kg. of body weight, while death may occur with a dose of 8 to 16 mg. per kg. of body weight. The toxicity of stilbestrol was not greater than an equal weight of estradiol benzoate as far as could be judged from effects in two dogs. A daily dose of 1 mg. of estradiol benzoate or stilbestrol given for 9 months, was not fatal. The dogs which were killed in a state of good health showed no toxic signs at autopsy.

Action of Androgens and Effect of Simultaneous Administration of Estrogens and Androgens

Daily doses of 5 to 10 mg. of testosterone or testosterone propionate were injected into 2 male dogs (*dogs* 12 and 10; tables 1, 2, 3) for 81 to 83 days without any deleterious effect on the blood, in contrast to the results with large doses of estrogens. Until the end the dogs remained healthy and the autopsy showed them to be quite normal. The prostate was larger than normal. Particularly noticeable was the good state of the heart muscle as compared with the friable condition in anemic dogs. The weight of the animals did not increase during the course of the experiment.

Dogs 19 and 23 (tables 1, and 3) received daily injections of 10 mg. of testosterone and 5 mg. of testosterone propionate, respectively, as well as 5 mg. of estradiol benzoate. Both dogs died with typical toxic symptoms, however. Thus, androgens did

not exert any protection against the action of the estrogens when administered in ratios of 2:1 or 1:1.

Action of Liver Preparations

Experiments were made to determine whether the anemia following administration of estrogens was caused by a lack of the liver factor. Four females were used. *Dogs* 9 and 11 (tables 1, 3) in which the number of red blood cells had decreased about 30% as the result of treatment with estradiol benzoate, were then given in addition a highly purified liver preparation[3] in daily injections of 4 to 8 cc., 1 cc. being equivalent to 50 gm. of liver substance. As will be seen from table 1, *dog* 11 which had received 14.2 mg. of estradiol benzoate per kg., died in spite of the liver therapy, while *dog* 9 with a dose of 6.66 mg. of estradiol benzoate per kg., remained alive. The anemia in this animal was very marked and even increased during the next 14 days after cessation of treatment with estradiol benzoate and in spite of continued treatment with liver preparation. The animal remained in a critical condition for 3 weeks after all treatment was discontinued (2 million R.B.C., 30% hemoglobin) and then slowly recovered from the anemia.

Dogs 16 and 17 (table 3) were treated for 10 days with 10 cc. of unpurified liver extract (1 cc. corresponding to 10 gm. of fresh liver; solid content about 14%) and then for 25 days with 10 cc. of liver extract plus the usual injection of 5 mg. of estradiol benzoate. At the same time, two control animals were injected with estradiol benzoate only. The two controls and *dog* 16 died, while *dog* 17 showed an anemia of 4.2 million R.B.C., 60% hemoglobin, 30.400 white blood cells, from which it eventually recovered. Our impression was that the degree of anemia depended on the amount of estrogens given and that, when this was sufficient, the liver factor did not significantly alter the course of the disease.

DISCUSSION

The course of the anemia, the differential count of the white blood cells and especially the frequent occurrence of granulocytopenia, point to a primary injury to the bone marrow. It is possible that large doses of estrogens induce alterations in the bone marrow which are incompatible with blood production. It is of interest that cases of granulocytopenia are most frequently met with in women. According to the monograph on this subject by Plum (16), the first 5 patients of Schultz (16) were women while Kracke and Parker (16) found in the literature 161 male (34.2%) and 310 female (65.8%) patients with granulocytopenia. Taussig and Schnoebelen (16) found 104 men (31.5%) and 226 women (68.5%). In Lichtenstein's report, of 27 cases 3 were men (11.1%) and 24 women (88.9%) (16), while in Plum's cases 17 were men (19.3%) and 71 women (80.7%). Whether this preponderance of granulocytopenia in females is simply fortuitous or dependent in part upon the estrogenic hormone remains a question.

Gardner (17), Gardner and Pfeiffer (18) Wentworth, Smith and Gardner (19) in mice and pigeons and Zondek (20) in young rats and chickens have observed partial or complete obliteration of the marrow space by endosteal bone deposition after long continued treatment with estrogens. Further investigation is required to determine whether the osteosclerotic process described by these workers is a contributing factor in the aplastic anemia observed in dogs.

It must be emphasized that the deleterious action of estrogens on the myelogenic system is limited to dogs. Other species of animals, such as mice, rats, guinea pigs are more resistant. Experiments in monkeys (21) indicate that these animals are more

[3] Pernaemon forte, Organon, Oss, The Netherlands.

resistant than dogs. Furthermore a daily dose of estradiol benzoate as high as 1 mg. given continuously for 9 months in dogs was not harmful. Larger doses of estrogens have also been used in man for therapeutic purposes without producing anemia.

SUMMARY

Aplastic anemia developed after a latent period of 1 to 3 weeks in normal dogs of both sexes and in castrated female dogs after treatment with large doses of estrone, estradiol benzoate or diethylstilbestrol. The most striking effect was a fall in the number of red blood cells, reticulocytes and thrombocytes and the occurrence of granulocytopenia after an initial leukocytosis with a shift to the left. In some cases other symptoms of granulocytopenia were observed such as necrotic lesions on the skin, throat and vagina. The bleeding time was prolonged and bleeding occurred under the skin, the mucous membranes of the intestinal and genital tracts and in various organs. At first the anemia was hyperchromic and later it became normo- or hypochromic.

When daily injections of 5 mg. in 1 cc. of olive oil were given until the onset of severe symptoms, the fatal total dose of estradiol benzoate lay between 8 and 16 mg. per kg. of body weight. The total quantity of estrogen tolerated depended upon the daily dose. A dose of 5 mg. given daily for 16 to 40 days was fatal for a dog of 10 kg., while a dose of 1 mg. daily for as long as 9 months was harmless.

Testosterone or testosterone propionate did not produce anemia even when amounts twice as large as those of the estrogen were given daily for several months. When the androgens were injected simultaneously with estradiol benzoate, they did not prevent the toxic effect of the estrogen.

The anemia following administration of estrogens was not prevented by the simultaneous injection of liver extracts.

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REFERENCES

1. Arnold, O., F. Holtz and H. Marx: *Naturwiss.* 24: 314. 1936.
2. Arnold, O., H. Hamperl, F. Holtz, K. Junkmann and H. Marx: *Arch. exp. Pathol. u. Pharmakol.* 186: 1. 1937.
3. Balo, J. v., and B. Purjesz: *Klin. Wchnsch.* 16: 1160. 1937.
4. Bareuther, A., and E. Schabbel: *Klin. Wchnsch.* 16: 1677. 1937.
5. Gardner, W. U.: To be published.
6. Tislowitz, R.: *Acta brev. neerl.* 8: 183. 1938.
7. Arnold, O.: *Klin. Wchnsch.* 18: 891. 1939.
8. Tislowitz, R.: *Acta brev. neerl.* 9: 15. 1939.
9. Scarborough, R. A.: *Yale J. of Biol. and Med.* 3: 359. 1930–31.
10. Wintrobe, M. M., H. B. Shumacker, Jr. and W. J. Schmidt: *Amer. J. Physiol.* 114: 502. 1936.
11. Hartman, C. G.: *Anat. Rec.* 35. suppl.: 13. 1927. *J. Mammal.* 9: 181. 1928. *Contr. Embryol. Carneg. Inst. Embryol.* 23: 1. 1932.
12. Bachman, C., J. B. Collip and H. Selye: *Proc. Roy. Soc. London s. B.* 117: 16. 1935.
13. Zuckerman, S., G. van Wagenen and R. H. Gardiner: *Proc. Zool. Soc. London s.* A. 108. Part 2. 1938.
14. Schultz, W.: *Deutsche med. Wchnsch.* 48: 1495. 1922.
15. Levi, H.: *Ned. Tijdschr. v. Geneesk.* 83: 2512. 1939.
16. Plum, P.: Clinical and experimental investigations in agranulocytosis. H. K. Lewis and Co., Ltd., London, 1937.
17. Gardner, W. U.: (a) *Proc. Soc. Exper. Biol. & Med.* 33: 104. 1935. (b) *Amer. J. of Anat.* 59: 459. 1936.
18. Gardner, W. U., and C. A. Pfeiffer: *Endocrinology* 23: 485. 1938.
19. Wentworth, J. H., P. K. Smith and W. U. Gardner: *Endocrinology* 26: 61. 1940.
20. Zondek, B.: *Folia clin. orient.* 1: 1. 1937.
21. Tyslowitz, R., and C. G. Hartman: *Endocrinology* 29: 349. 1941.
22. Castrodale, D., O. Bierbaum, E. B. Helwig, and C. M. MacBryde: *Endocrinology* 29: 363, 1941.
23. Crafts, R. C.: *Endocrinology* 29: 606. 1941.

EFFECT OF SEX AND GONADOTROPIC HORMONES UPON THE BLOOD PICTURE OF THE RAT

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THE ANEMIA FOLLOWING HYPOPHYSECTOMY in rats now seems to be well established (1, 2, 3). Reticulopenia (4, 5) and bone marrow hypoplasia (6, 3) have also been noted in hypophysectomized rats. Conversely, it has been found possible to produce reticulocytosis and polycythemia in normal and hypophysectomized rats by means of treatment with anterior pituitary extracts (7, 8). Those interested in the problem apparently agree that anterior pituitary substances in some way stimulate erythrocyte formation in the bone marrow.

The manner in which such stimulation is produced is as yet unknown. Flaks and his coworkers (7, 8), without critical evidence, have attributed it to a special fraction or 'hemotropic hormone' such as Moehlig and Bates (9) had postulated upon the basis of clinical evidence. This idea has been tentatively accepted by Overbeek and Querido (6). Vollmer and his coworkers (3) however, have stressed the possibility that the stimulation is effected through the general metabolic activities of the pituitary. More recently Meyer et al. (10), having found that thyroxin, thyrotropic, adrenotropic and growth hormones bring about reticulocytosis in hypophysectomized rats, have concluded that these principles operate through their effects on metabolism. Older work had indicated that removal of the thyroid (11, 12) and possibly the malfunctioning of the adrenal cortex (13) were accompanied by anemia.

Estrogens, also, have been observed to produce anemia in dogs (14, 15) and to cause fluctuations in the red cell count when given *in utero* in the rat (16). Low erythrocyte counts have been reported in normal female rats injected with pregnant mare serum (17). Androgens, on the other hand, have been reported to increase the red cell count in rabbits (18) and in humans (19). These apparently opposite effects of male and female hormones bring to mind the fact that the blood of the human male contains, on the average, 700,000 more erythrocytes per cu. mm. than that of the female (20). Similar differences have been noted in fowl (21–24), in pigeons (25), in rabbits (26), in cats (27), in rats (28), in mice (29) and in other laboratory and domestic animals (30). Almost invariably it is the male animal which has the higher count. These facts raised the question whether such sex differences in the blood picture might be due to actions of the sex hormones upon the hemopoietic system. The present work is an investigation of this question.

MATERIALS AND METHODS

Young, mature rats of 150 to 230 grams, from an inbred colony, were used. They were kept in a constant temperature room and fed a balanced laboratory ration, in-

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cluding fresh carrots and lettuce. In addition, all hypophysectomized animals received 20% sucrose for drinking water. Ether was used in all operations, and hypophysectomy was performed by the parapharyngeal approach. Incompletely hypophysectomized animals were either eliminated from, or are clearly indicated in, the data.

The usual hematological and histological techniques were utilized in the manner previously described (3). In these experiments, however, a Hellige hemometer giving readings in grams per hundred cc. of blood was used for the hemoglobin determinations, and one investigator made the duplicate erythrocyte and leucocyte counts. These, as before, were made with standardized pipettes and were required to show an agreement of $\pm 4\%$ and $\pm 10\%$ respectively. Reticulocytes were counted on wet smears stained with brilliant cresyl blue. For histological examination of the bone marrow, right femurs cut at both ends were fixed in Bouin's solution, decalcified in phloroglucin nitric acid, and sectioned in paraffin. The sections were stained with Harris' hematoxylin-eosin.

The first group of data consists of blood cell counts made upon unoperated animals and castrate females. Some of these were untreated; the rest, at the time the counts were made, had been treated for 6 to 8 weeks with pregnant mare serum, testosterone propionate, estradiol benzoate or sesame oil. The castrated animals were all of about the same age and had been operated upon 2 months before the experiments were begun.

The second phase of the work was experimental, complete blood determinations being made upon groups of normal males and females treated with pregnant mare serum. Counts were made before treatment and weekly for 2 months after treatment began.

In the third phase, hypophysectomized animals were used, to eliminate complications arising from the interaction between the hormones injected and the animals' own pituitary glands. Unless otherwise stated the animals underwent operation 6 weeks before treatment started, and therefore showed the characteristic post-hypophysectomy anemia in the first recorded counts. Subcutaneous injections were made daily, in the amount indicated below for each group. The preparations[2] used included pregnant mare serum extract, pregnancy urine extract, estradiol, testosterone, progesterone, crude anterior pituitary extract, sesame oil, normal horse serum, and crude muscle extract. The crude pituitary extract was a saline infusion of beef anterior pituitary ground in clean sand, centrifuged, and stored in a frozen state until use (31). The crude muscle extract was made in the same way with fresh beef muscle.

RESULTS

Normal rats and castrate female rats, untreated or treated with sex and gonadotropic hormones. Since the data from these preliminary observations are given in table 1, they will be described only briefly. The erythrocyte counts of normal male and female rats of the colony show a mean difference of 400,000 cells per cu. mm. This difference between the mean counts of normal males and females is accentuated after 2 months' treatment with 15 R.U. of PMS, with the male count more than two million cells per cu. mm. higher than that of the female. Hemoglobin also increased significantly in the treated males.

The erythrocyte counts of females, 4 months after castration, averaged one mil-

[2] Testosterone propionate (Oreton), corpus luteum hormone (Proluton), alpha-estradiol benzoate (Progynon-B) and pregnancy urine hormone (Pranturon) were supplied by Dr. Erwin Schwenk of the Schering Corporation. The pregnant mare serum preparations (Veterinary Gonadin and Human Gonadin) were supplied by Mr. Donald Wonder of the Cutter Laboratories.

lion cells per cu. mm. more than those of normal females. Included in these results are the counts of 13 untreated castrates, 3 treated daily for 2 months with 0.1 cc. of sesame oil, and 8 treated with 10 R.U. of PMS daily for 2 months. The range and mean count for similar animals which had been injected daily for 2 months with 0.5 mg. of testosterone were even higher, while the figures for castrates injected with 10 R.U. estradiol for 2 months were considerably lower than those of untreated castrates.

TABLE 1. ERYTHROCYTE COUNTS OF NORMAL AND CASTRATE ANIMALS, UNTREATED OR TREATED WITH SEX AND GONADOTROPIC HORMONES

Animals	Treatment	RBC—Millions/cu. mm. M±S.E.	Range
25 Normal Males	None	8.6±0.1	7.9– 9.6
6 Normal Males	Preg. Mare Serum	10.3±0.3	9.7–10.8
50 Normal Females	None	8.2±0.1	7.4– 9.1
8 Normal Females	Preg. Mare Serum	7.6±0.3	7.3– 8.6
16 Castrated Females	None, or Sesame	9.3±0.2	8.2–10.6
8 Castrated Females	Preg. Mare Serum	9.0±0.3	8.1–10.2
5 Castrated Females	Testosterone	9.8±0.4	8.9–11.1
5 Castrated Females	Estradiol	7.9±0.3	7.6– 8.2

Normal rats treated with pregnant mare serum. The effects of PMS on normal animals were followed more closely in the next experiment. As the preliminary observations led one to expect, the erythrocyte counts of 5 males treated daily with 15 R.U. of PMS showed weekly increases (fig. 1), the mean[3] rising from 8.3 ± 0.4 before treatment to 9.8 ± 0.3 at 8 weeks. Opposed to this gain of 18% in red blood cells in the males, 5 normal females injected with 10 R.U. of PMS showed declining erythrocyte numbers (fig. 2) with a mean loss of 7% in the sixth week. When a larger dosage (i.e. 15 R.U.) was used upon normal females, the maximum mean loss occurred earlier, in the third week; beyond this time the counts tended to return toward normal. The erythrocyte counts of 4 normal males and 5 normal females injected with 0.3 and 0.2 cc. of normal horse serum respectively showed increases which were probably insignificant (4% maximum). In all these groups no distinct trend was seen in hemoglobin, total leucocyte or differential determinations; and only the males treated with PMS showed significantly increased reticulocyte counts.

Hypophysectomized rats treated with crude beef pituitary. Five hypophysectomized male rats were injected daily with 1.0 cc. of crude beef pituitary extract, from the fifth week after operation. The fluctuations which ensued in the erythrocyte counts were so great that the use of averaged counts would be misleading. In general, the course was as follows: a rapid drop in the count for the first week or two, then a series of sharp fluctuations in the course of which, in the third month, normal values were temporarily attained. Hemoglobin determinations fluctuated similarly. During the early weeks there was a strong reticulocytosis, with an average of 13% on the twelfth day, followed by a gradual decline to an average of 1% at the end of two months of treatment. In contrast with the blood changes, the average body weight increased linearly during treatment from 135 to 225 gm. at the end of the second month, and then levelled off. The blood of 5 other hypophysectomized animals, injected for 5 to 8 weeks with a different batch of similarly prepared extract, was found to contain normal numbers of erythrocytes. The blood of 5 hypophysectomized rats

[3] Means for groups of four or less are given with the average deviation; of five or more, with the standard error.

injected with 1.0 cc. daily of crude muscle extract showed neither the acute changes in erythrocyte, hemoglobin and reticulocyte values, nor the approximate attainment of normal red cell count revealed by animals injected with the pituitary extract.

Hypophysectomized rats treated with pregnant mare serum. The erythropoietic response of males and females was sharply differentiated when PMS was injected beginning with the sixth week after hypophysectomy (fig. 3). Four females, with a mean erythrocyte count at this time of 6.0 ± 0.5 million per cu. mm. and a mean

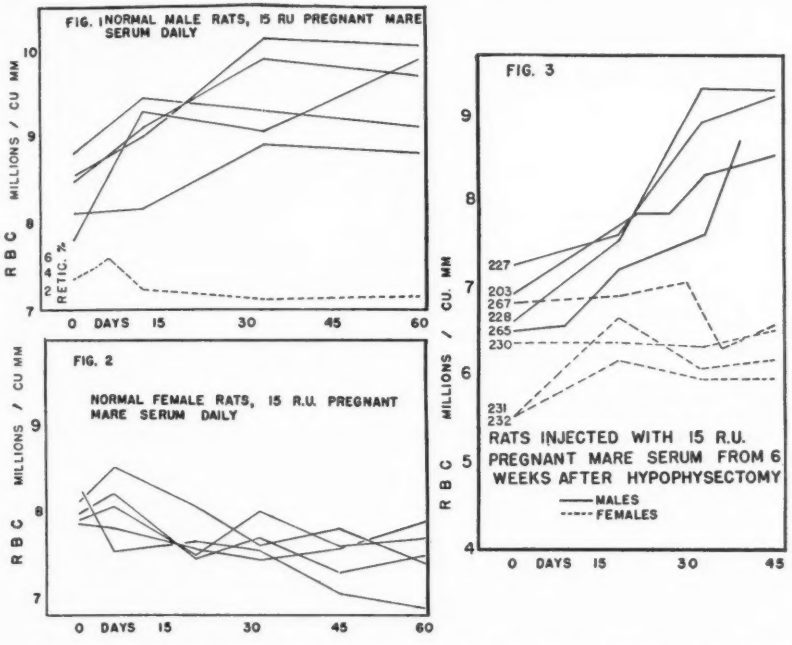

Fig. 1, Fig. 2. Erythrocyte counts of unoperated male and female rats injected daily with 15 r.u. of pms. Fig. 3. Erythrocyte counts of hypophysectomized rats injected daily with 15 r.u. of pms from the 6th week after operation.

hemoglobin value of 14.3 ± 1.5 gm. per hundred cc., underwent little change from these values during 6 weeks of treatment. In the fifth week of treatment the mean count was 6.1 ± 0.3 million per cu. mm. and the mean hemoglobin value of 13.8 ± 0.8 grams. The 4 males, on the contrary, showed rapidly increasing values. The mean red cell count increased from 6.8 ± 0.3 million per cu. mm. at the beginning of treatment to 8.9 ± 0.3 in the fifth week, while the hemoglobin rose from 13.1 ± 1.1 to 16.1 ± 1.2 gm. per hundred cc. Thus with the same treatment (15 r.u. daily of PMS) the females remained at their low post-hypophysectomy levels while the males gained 31% in erythrocytes and 19% in hemoglobin. Three of the males also gained 15% in body weight while the other male and the females were unchanged. Leucocytic changes showed no apparent tendency. The testes of the males became palpable after a week of treatment and reached normal size at about three weeks. The ovaries of the females contained numerous follicles, and the uteri were normal in appearance. The red blood cell values of 3 hypophysectomized males and 3 hypophysectomized females

injected daily with 0.3 cc. of normal horse serum from the sixth week after operation showed insignificant fluctuations.

Hypophysectomized rats treated with pregnancy urine hormone. The hemopoietic response of hypophysectomized rats to PU hormone was small. The low post-hypophysectomy count of 5 animals (3 males, 2 females) showed increments of about one million cells per cu. mm. in the course of 6 weeks' treatment with 20 I.U. daily of this hormone, and even these small gains were irregular. No differences in count appeared between males and females with this treatment.

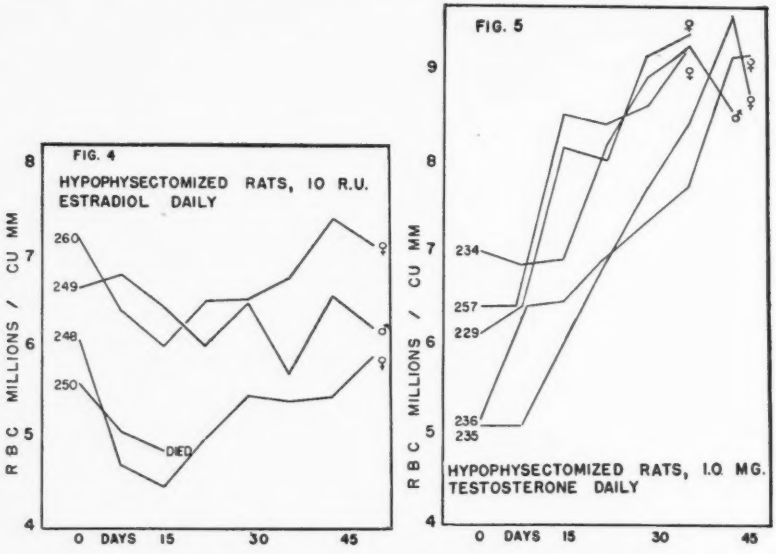

Fig. 4, Fig. 5. ERYTHROCYTE COUNTS OF HYPOPHYSECTOMIZED RATS injected daily with 10 R.U. of estradiol benzoate, or 1.0 mg. of testosterone propionate from the 8th week after operation.

Hypophysectomized rats treated with estradiol in sesame oil. It was found that hypophysectomized rats were usually able to tolerate a dose of 10 R.U. daily of estradiol in sesame oil. One female died after 2 weeks' treatment, but 2 females and a male survived for 6 weeks, at which time they were sacrificed for their marrows. The erythrocyte counts (fig. 4) showed an early decline of about one and a half million cells per cu. mm., with a gradual return toward the original counts. Hemoglobin varied little, and the reticulocyte percentages remained low.

Hypophysectomized rats treated with testosterone in sesame oil. Most striking changes were encountered in the blood picture of 4 females and a male injected with 1.0 mg. daily of testosterone. Their mean erythrocyte count 8 weeks after hypophysectomy was 6.0 ± 0.4 million per cu. mm., but after 5 weeks of treatment it had become 9.1 ± 0.3, an increase of 51% (fig. 5). During this period the hemoglobin increased from 13.3 ± 0.8 to 15.6 ± 0.3, or by 17%. The reticulocyte count in most instances was elevated at the end of the first and second weeks, but declined thereafter (fig. 6). The average gain in weight for these 5 animals, which remained in apparent good condition until the end of the experiment, was 14 gm. or 8%. Two other animals which died before the end of the first month and one which was eliminated because

of disease showed similar marked increases in erythrocyte number during the early weeks of treatment.

Hypophysectomized rats treated with progesterone in sesame oil. The results obtained through the injection of 1.0 mg. of progesterone daily for 6 weeks were inconsistent. Three females (one incompletely hypophysectomized) showed apparently significant augmentation of the red cell count, while two other females and a male did not. Hemoglobin was fairly constant, body weight increased slightly, and there were some sporadic high reticulocyte counts.

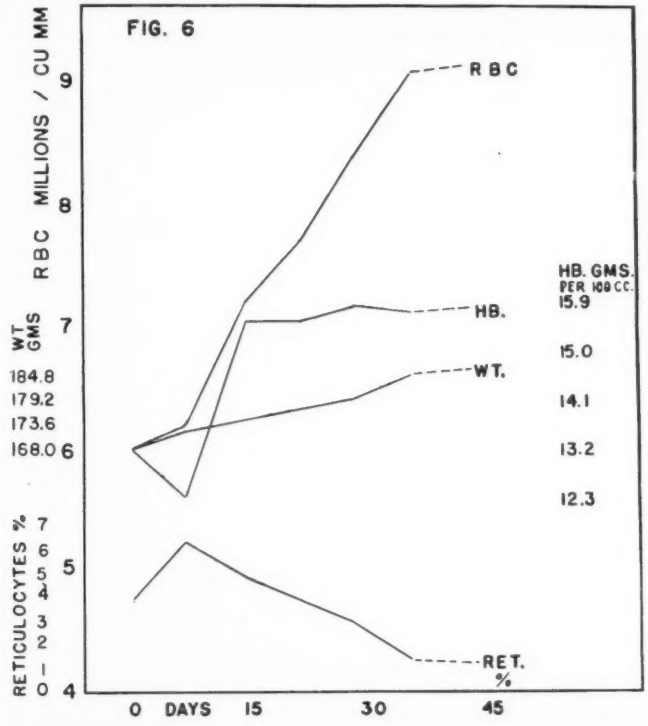

Fig. 6. MEAN ERYTHROCYTE COUNTS, HB. VALUES, RETICULOCYTE PERCENTAGES AND BODY WT. OF 5 HYPOPHYSECTOMIZED RATS, injected daily with 1.0 mg. of testosterone propionate from the 8th week after operation. The values, except for reticulocytes, are pro-rated so as to coincide at the time of the first injection.

Hypophysectomized rats treated with sesame oil. When hypophysectomized rats were injected with 0.1 cc. of sesame oil daily (the amount used with estradiol, testosterone and progesterone in the above experiments), death usually occurred within a few weeks. Of 8 females, 3 died after one week and one each in the second, third, fifth and sixth weeks; the single survivor was found to be incompletely hypophysectomized. In two instances there was a sudden increase in the erythrocyte count just before death; in the others there were insignificant changes, mostly decreases in cell number.

Bone marrow histology. Examination of sections of bone marrow taken from representative animals in each series revealed, in general, that androgens stimulate and es-

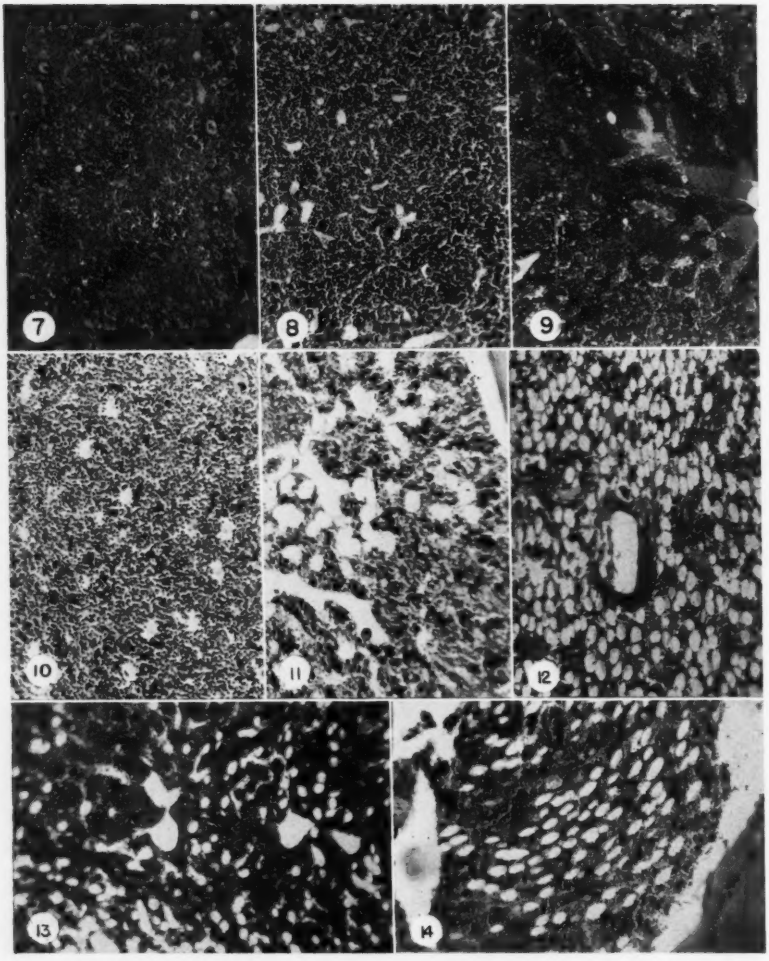

Fig. 7. Bone marrow of hypophysectomized male rat injected daily for 6 weeks with 1.0 mg. of testosterone propionate, from the 8th week after operation. ×77.

Fig. 8. Bone marrow of hypophysectomized female rat injected daily for 6 weeks with 1.0 mg. of testosterone propionate, from the 8th week after operation. ×77.

Fig. 9. Bone marrow of hypophysectomized male rat injected daily for 6 weeks with 15 R.U. of PMS, from the 6th week after operation. ×77.

Fig. 10. Bone marrow of adult, unoperated, untreated male rat. ×77. Marrow from a normal female has the same appearance.

Fig. 11. Bone marrow of untreated female rat, 4 months after hypophysectomy. ×77. Marrow from a hypophysectomized male has the same appearance.

Fig. 12. Bone marrow of hypophysectomized female rat injected daily for 6 weeks with 10 R.U. of estradiol benzoate, from the 8th week after operation. ×77.

Fig. 13. Bone marrow of hypophysectomized female rat injected daily for 6 weeks with 15 R.U. of PMS, from the 6th week after operation. ×77.

Fig. 14. Bone marrow of unoperated female rat injected daily for 60 days with 10 R.U. of PMS. ×77.

trogens depress erythropoiesis. Thus in both hypophysectomized males and females injected with testosterone, the post-hypophysectomy hypoplasia was considerably repaired (fig. 7, 8). Erythroblasts and megakaryocytes increased in number, and vacuolization was less marked. The marrows of hypophysectomized males injected with pregnant mare serum revealed a similar picture (fig. 9). The marrows of normal males treated with PMS were indistinguishable from those of normal untreated males (fig. 10).

The hypoplasia apparent in the marrows of hypophysectomized animals (fig. 11) seemed to be increased by estradiol injections (fig. 12), vacuolization being more marked and the numbers of red cell progenitors somewhat reduced. Similar but less marked alterations were observed in the bone marrows of hypophysectomized females treated with PMS (fig. 13). In unoperated females, continued injections of PMS resulted in a definite hypoplasia of the marrow (fig. 14).

Crude anterior pituitary extract and progesterone treatment seemed to repair only partially the post-hypophysectomy marrow aplasia.

DISCUSSION

The experiments described indicate that androgens increase while estrogens decrease the erythrocyte count of rats. This is true whether the sex hormones are injected directly or are produced by gonadotropic stimulation. Thus, for example, the normal sex difference in the red cell count can be markedly increased by injecting identical amounts of the same gonadotropic agent (e.g., PMS) in the two sexes. Since the castrate females showed no erythrocyte response to gonadotropic hormones, it may be inferred that the changes occurring in non-castrates are due to endogenous production of their respective hormones. The androgen effect appears to be especially strong, since 29 animals (normal and hypophysectomized males treated with PMS, castrate females, and hypophysectomized animals treated with testosterone) without exception showed counts well above normal. The estrogen effect appeared definitly in the lowered counts of 25 animals (normal females treated with PMS, castrate females and hypophysectomized animals treated with estradiol) but not in the almost unfluctuating low counts of 7 hypophysectomized females treated with PMS.

It may now be asked whether these changes in erythrocyte number are a reflection of bone marrow activity or of varying plasma volume. The bone marrow sections are consistent in support of the first possibility: a normal or repaired condition in androgen influenced animals, hypoplasia in the estrogen influenced animals, including unoperated females treated with PMS. Secondly, reticulocytosis was most marked in the hypophysectomized animals injected with testosterone and in males injected with PMS, while the estrogen-treated animals revealed no reticulocytosis. Finally, hemoconcentration is rendered improbable by a comparison of the hemoglobin and erythrocyte changes in the androgen influenced groups. While hemoglobin increased 19% in the blood of hypophysectomized males treated with PMS, the red cell number increased 31%; in hypophysectomized animals treated with testosterone hemoglobin increased only 17% while the erythrocytes increased by 51%. In the latter group the increases in hemoglobin were confined to the first two weeks, while cell numbers continued to increase for another month (fig. 6). It would thus appear that androgens are able, in some way, to increase red cell proliferation in the marrow without proportionately increasing the elaboration of hemoglobin. In this connection it may be recalled that testosterone under some conditions stimulates somatic growth (32, 33), while the depressing effects of excess estrogens on somatic growth are well known.

It is interesting to note that PU hormone has negligible effects upon the erythrocyte and hemoglobin levels of hypophysectomized rats. This was expected, since this

hormone furnishes only incomplete replacement for natural gonadotropins (34), while PMS affords more complete replacement (35). Less expected, however, was the finding that whole beef anterior pituitary extract required two months to bring the cell count to normal levels and failed to keep it there. This might be attributed to the fact that the extract was gonadotropically weak, but Perla (36) has observed also that such extracts may cause the hypertrophy of RE elements in the spleen, with evidence of red cell destruction. Thus some crude, non-homologous extracts perhaps do cause blood destruction, as Querido and Overbeek have claimed (37) but this does not mean that the hypophysis normally exerts its hemopoietic influence by way of blood destruction. The question of mechanism, in fact, requires new experiment and study, the present evidence indicating that the answer is not a simple one.

Flaks, Himmel and Zlotnick (8) have produced bone marrow hyperplasia and polycythemia in hypophysectomized rats by means of a certain AP fraction, and have therefore spoken of a 'hemotropic hormone.' This fraction was said to be free of growth, thyrotropic and gonadotropic hormone. Yet Mayer et al. (10) have produced polycythemia in hypophysectomized animals by means of the combined stimuli of reduced oxygen tension and growth hormone, and have prevented anemia in similar animals with thyroxin and with thyrotropic hormone. We have restored the blood count completely and the hemoglobin titer partially in hypophysectomized animals with testosterone, and, in males, with PMS. Thus all of the principles removed from the Flaks preparation have been shown to have, separately, strong erythrocytopoietic properties. It is therefore reasonable to think that the anemia following hypophysectomy is due at least in part to the atrophy of certain endocrine glands after this operation. Since numerous disruptions in metabolism occur at this time it is not improbable that these, rather than the loss of some specific bone marrow stimulator, are responsible for the anemia.

We are unable to explain the fact that control hypophysectomized animals injected with sesame oil only usually survived for only a short time. Toxicity of this substance in adrenalectomized animals has been reported (38). Inasmuch as hypophysectomized animals injected with testosterone-in-sesame showed increased red cells while those injected with estradiol-in-sesame showed decreases, it seems likely that the hormones rather than the sesame oil were responsible for these changes.

The failure to obtain positive results with regard to the leucocytes is by no means proof that they are unaffected by hormone therapy. There is so much variation in the initial counts that many more cases would be needed to reveal any tendencies present. Another difficulty is the lability of the leucocytic elements, which respond readily to foreign substances and to minor changes in the condition of the animal.

SUMMARY

It has been shown that testosterone propionate raises the erythrocyte counts of hypophysectomized male and female rats from anemic to a high normal level, and partially restores the hemoglobin content of the blood of such animals. This hormone also causes a slight but significant increase in the erythrocyte counts of castrated females. Pregnant mare serum has the same effect as testosterone on the red cell counts of hypophysectomized males, and significantly raises the erythrocyte counts of normal untreated males. Reticulocytosis and bone marrow hyperplasia are usual in these androgen-influenced groups. Therefore it is thought that the male hormone causes elevation of the red cell count, as a result of increased erythropoiesis.

Estradiol benzoate lowers the erythrocyte count in castrated females and temporarily intensifies the post-hypophysectomy anemia. Pregnant mare serum lowers the erythrocyte count of unoperated females but does not alter the counts of cas-

trated females or the low counts of hypophysectomized females. Bone marrow hypoplasia is evident in the estradiol treated animals and in unoperated females treated with pregnant mare serum; reticulocyte counts in these animals were generally low. It is therefore thought that estrogens tend to lower the erythrocyte counts of rats, through diminished bone marrow activity.

In general, hemoglobin changes are less marked than those in red cell number. The leucocytic elements apparently are not affected by the hormones used in these experiments.

PU hormone appears to have no effect upon the blood picture of hypophysectomized rats, and progesterone has given inconsistent results. Normal horse serum, sesame oil, and crude beef muscle extract have no erythropoietic effects.

Since the erythropoietic activities of the bone marrow of hypophysectomized animals can be affected by androgens, in addition to other hormones, as shown by other workers, it is felt that the anemia following hypophysectomy is due in part to a disturbance in metabolism resulting from atrophy of various endocrine glands. It is also thought that the gonadal secretions are responsible for the sex difference in normal red cell count encountered in many species of animals.

We are greatly indebted to Dr. Harry A. Charipper for advice and encouragement, and to Dr. Irving Levenstein for performing the surgical operations.

REFERENCES

1. Meyer, O. O., G. E. Stewart, E. W. Thewlis and H. P. Rusch: *Folia haemat.* 57: 99. 1937.
2. Parodi, S.: Thesis, Buenos Aires, 1937. (*Brit. Chem. & Physiol. Abst. A.*, III: 473. 1939).
3. Vollmer, E. P., A. S. Gordon, I. Levenstein and H. A. Charipper: *Endocrinology* 25: 970. 1939.
4. Stewart, G. E., R. O. Greep and O. O. Meyer: *Proc. Soc. Exper. Biol. & Med.* 33: 112. 1935.
5. Overbeek, G. A.: *Arch. internat. de pharmacodyn. et de thérap.* 54: 340. 1936.
6. Overbeek, G. A., and A. Querido: *Arch. internat. de pharmacodyn. et de thérap.* 60: 105. 1938.
7. Flaks, H., I. Himmel and A. Zlotnick: *Presse méd.* 45: 1261. 1937.
8. Flaks, J., I. Himmel and A. Zlotnick: *Presse méd.* 46: 1506. 1938.
9. Moehlig, R. C., and G. S. Bates: *Arch. Int. Med.* 51: 207. 1933.
10. Meyer, O. O., E. W. Thewlis and H. P. Rusch: *Endocrinology* 27: 932. 1940.
11. Kishi, K.: *Virchows Arch. f. path. Anat.* 176: 260. 1904.
12. Kunde, M. M.: *Am. J. Physiol.* 76: 225. 1926.
13. Hartman, F. A., C. W. Greene, B. D. Bowan and G. W. Thorn: *J.A.M.A.* 99: 1478. 1932.
14. Balo, J. V., and B. Purjesz: *Klin. Wchnschr.* 16: 1150. 1937.
15. Barenther, A., and E. Schabbel: *Klin. Wchnschr.* 16: 1677. 1937.
16. Arnold, O. H., P. Gumbrecht and A. Loesser: *Arch. f. exper. Path. u. Pharmakol.* 191: 192. 1938.
17. Gordon, A. S., I. Levenstein and H. A. Charipper: *Proc. Am. Physiol. Soc.* p. 68. 1940.
18. del Zoppo, R.: *Clin. med. ital.* 2: 113. 1937.
19. Albeaux-Fernet and Lefebvre: *Sang.* 13: 926. 1939.
20. Ponder, E.: The Mammalian Red Cell and the Properties of Hemolytic Systems. P. 3. Verlag von Gebrüder Borntraeger, Berlin. 1934.
21. Fritsch, G.: *Pflügers Arch. f. d. ges. Physiol.* 181: 78. 1920.
22. Welsch, W.: *Pflügers Arch. f. d. ges. Physiol.* 198: 37. 1923.
23. Blacher, L. J.: *Biol. generalis* 2: 435. 1926.
24. Chaudhuri, A. C.: *Proc. Roy. Phys. Soc.* 21: 109. 1926.
25. Riddle, O., and P. Braucher: *Am. J. Physiol.* 108: 554. 1934.
26. Rosahn, P. D., L. Pierce and Ch'uan-K'uai Hu: *J. Exper. Med.* 60: 687. 1934.
27. Lewis, L. A.: *Endocrinology* 28: 821. 1941.
28. Adams, A. E., and F. Shevket: *Physiol. Zoöl.* 2: 181. 1929.
29. Kamenoff, R. J.: *Proc. Soc. Exper. Biol. & Med.* 36: 411. 1936.
30. Scarborough, R. A.: *Yale J. Biol. & Med.* 3: 63; 169; 1930. 267; 359; 431; 1931.
31. Young, F. G.: *Biochem. J.* 32: 513–523. 1938.
32. Papanicalaou, G. N., and E. A. Falk: *Science* 87: 238. 1938.
33. Rubinstein, H. S., and M. L. Solomon: *Proc. Soc. Exper. Biol. & Med.* 44: 442. 1940.
34. Collip, J. B., H. Selye and D. L. Thompson: *Nature* 131: 56. 1933.
35. Evans, H. M., K. K. Korpi, M. E. Simpson and R. I. Pencharz: *Univ. of Cal. Pub. in Anat.* No. 10. 1: 275. 1936.
36. Perla, D.: *J. Exper. Med.* 63: 599. 1936.
37. Querido, A., and G. A. Overbeek: *Arch. internat. de pharmacodyn. et de thérap.* 61: 475. 1939.
38. Bruce, R. A., and C. E. Tobin: *Endocrinology* 27: 956. 1940.

DIABETOGENIC EFFECT OF STILBESTROL IN FORCE-FED NORMAL AND PARTIALLY DEPANCREATIZED RATS

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THIS REPORT EXTENDS PRELIMINARY OBSERVATIONS (1, 2) that under certain experimental conditions the administration of stilbestrol to rats causes hyperglycemia and glycosuria. This substance administered to partially depancreatized, non-glycosuric rats on a constant medium carbohydrate diet produced a marked glycosuria in all of a large series of cases. Other estrogens were found to have this property and massive doses of androgens were weakly effective. In normal non-glycosuric rats on a constant high carbohydrate diet stilbestrol produced a mild, temporary glycosuria in the majority of a large series of cases. Dolin, Joseph and Gaunt (3) have reported that estrogens are effective in intensifying the glycosuria of the partially depancreatized ferret.

METHODS

Male rats of the Sprague-Dawley strain were used. Normal and partially depancreatized animals were studied. Partial pancreatectomy was carried out at a body-weight of 45 to 80 gm. In one series of animals all of the pancreas was removed except that portion between the bile duct and the duodenum. The remaining portion represented an average of about 5% of the total weight of the pancreas. These rats are identified as 95% depancreatized. In the second series of rats all of the pancreas was removed from the greater omentum and spleen and all of the pancreas lying within the duodenal loop was left intact. The remaining portion represented an average of about 25% of the total weight of the pancreas. These rats are identified as 75% depancreatized. These values are used only for reference to experimental groups and do not provide an accurate index to the relative functional capacity of the pancreatic remnants.

All of the experimental animals were maintained on a diet of Purina dog chow until they had reached a weight of 275 to 300 gm. They were then placed in metabolism cages and maintained on a fluid diet administered by stomach tube each morning and night. The two diets used were made up of the following constituents:

Diet	*Medium Carbohydrate*	*High Carbohydrate*
Egg albumin	200 gm.	200 gm.
Cellu Flour	120 gm.	120 gm.
Osborne and Mendel salt mixture	40 gm.	40 gm.
Vitamin preparation (Vi-Penta)	10 cc.	10 cc.
Wheat germ oil	10 cc.	10 cc.
Mazola oil	10 cc	10 cc.
Starch	200 gm.	540 gm.
Dextrin	100 gm.	260 gm.
Sucrose	100 gm.	265 gm.
Butter (85% fat)	345 gm.	
Water to make total volume of	2,000 cc.	2,000 cc.

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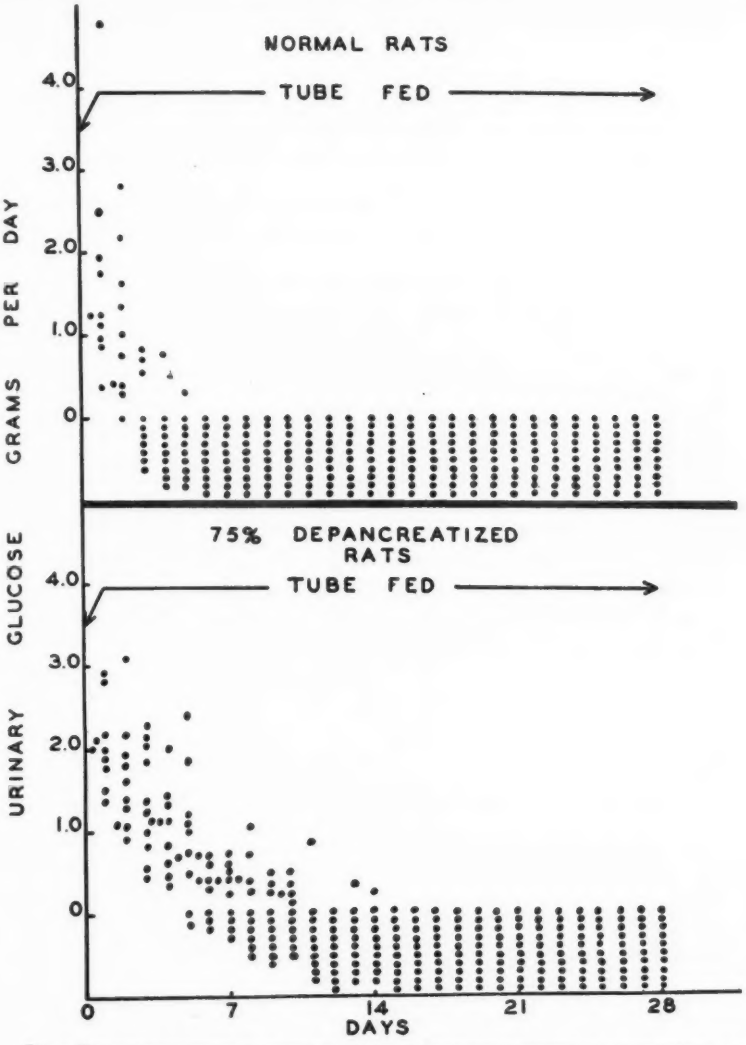


Fig. 1. Duration of period of glycosuria in rats following beginning of force-feeding a constant amount of high carbohydrate diet. These values for urinary glucose were obtained after the completion of a short adaptation period (see text) and immediately following the beginning of feeding 26 cc. of the diet per day.

The basal constituents of the two diets are the same and they are approximately isocaloric. Each rat received 26 cc. of diet each day. For each rat the daily intake of nitrogen was approximately 420 mg.; the total available carbohydrate of the medium diet was approximately 6.5 gm.; and the total available carbohydrate of the high carbohydrate diet was approximately 15 gm. These values are based on analyses of the diet and assume that the maximum formation of glucose from the protein of the diet does not exceed a glucose: nitrogen ratio of 3.6.

At the beginning of force-feeding of either normal or partially depancreatized rats it is necessary to begin administering the diet in small amounts to prevent the development of diarrhea and 'food-shock.' The normal rats were brought to a full feeding on the 3rd day, the 75% depancreatized rats were brought to a full feeding on the 6th day and the 95% depancreatized rats were brought to a full feeding on the 8th day. Following the early development of tolerance to the diet such rats can be maintained for indefinite periods of time.

All of the test substances were administered in divided doses each morning and night. The estrogens and androgens were in sesame oil solution of a total daily volume

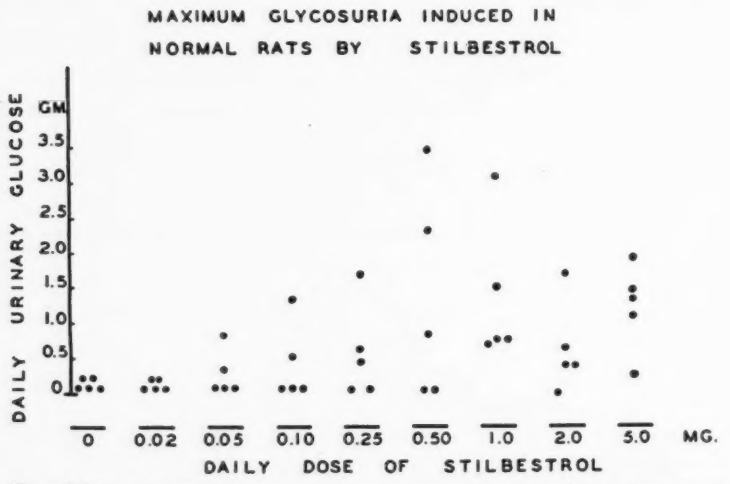


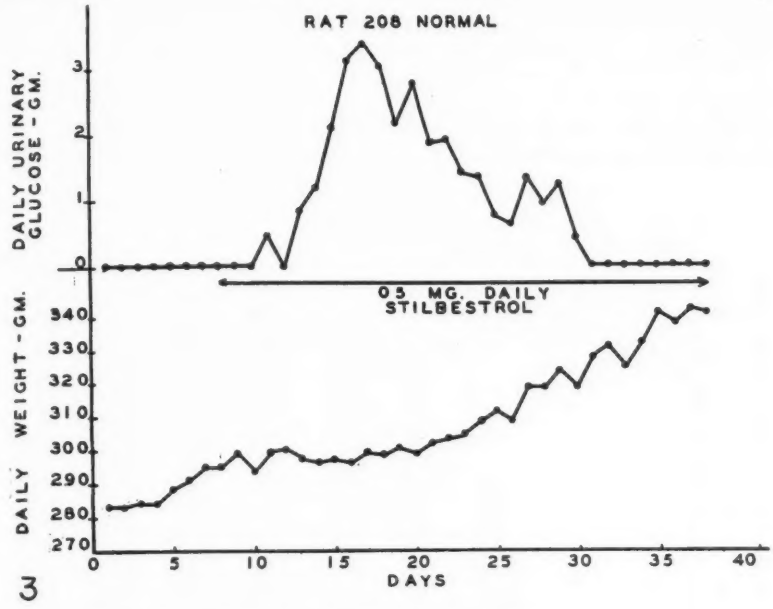
Fig. 2. Maximum values for urinary glucose in normal rats in relation to dosage of stilbestrol. The control rats in this series were injected with sesame oil. Each rat was fed 26 cc. of the high carbohydrate diet daily. The duration of the period of glycosuria in this series was 2–36 days.

of 0.5 cc. The formaldehyde was in water solution. The carbon tetrachloride was diluted with sesame oil. All solutions were administered subcutaneously. The water was given intraperitoneally. Twenty-four-hour specimens of urine were collected at the same hour each day and preserved with toluene. The methods used in analyses have been described by Evans (4).

EXPERIMENTS AND RESULTS

All of the normal rats and all of the 75% depancreatized rats were maintained on the high carbohydrate diet. Following the beginning of the administration of 26 cc. of diet per day most of the animals of each group excreted some glucose for a short period but adaptation soon occurred and the urine was thereafter free of glucose. Figure 1 shows the extent and duration of the spontaneous glycosuria of 10 normal and ten 75% depancreatized rats which were selected by random sampling. The duration of the period of spontaneous glycosuria was greater in the partially depancreatized than in the normal animals. Many of the rats which were 95% depancreatized continued to have a spontaneous glycosuria on either the medium diet or the high carbohydrate diet.

Experiment 1 was a study of the relationship between dosage of stilbestrol and the glycosuric response of normal rats. The injections were begun after the animals



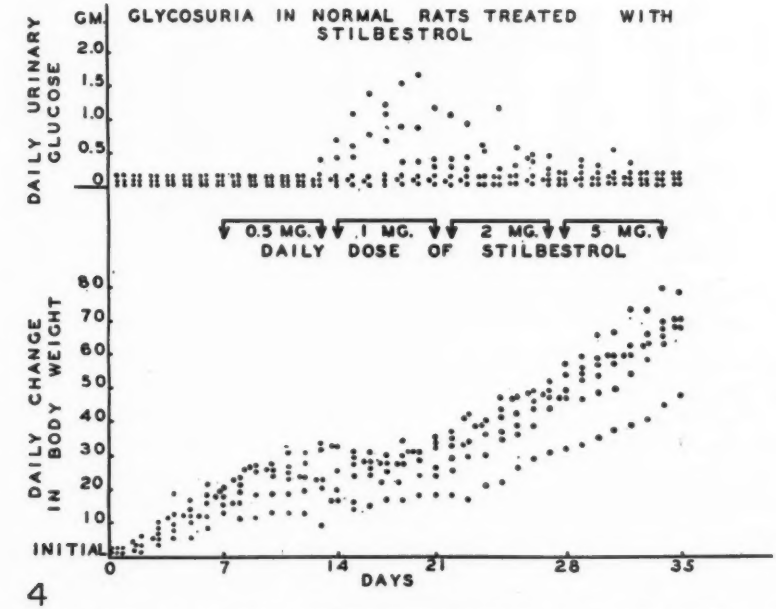


Fig. 3. Extent and duration of stilbestrol diabetes in normal rat. This animal was fed 26 cc. of the high carbohydrate diet daily. Fig. 4. Failure of increased dosage of stilbestrol to maintain diabetic state in normal rats. These rats were each fed 26 cc. of the high carbohydrate diet daily.

had been free from glycosuria for a minimum period of 10 consecutive days. Injections were continued for 14 days in those rats which did not develop glycosuria. In those rats which did show a glycosuric response to stilbestrol the injections were continued beyond the period of glycosuria until the urine was free of sugar for 7 consecutive days. Figure 2 contains the data on dosage and the maximum amount of glucose excreted in any 24-hour period for each animal. Figure 3 shows the course of the diabetic

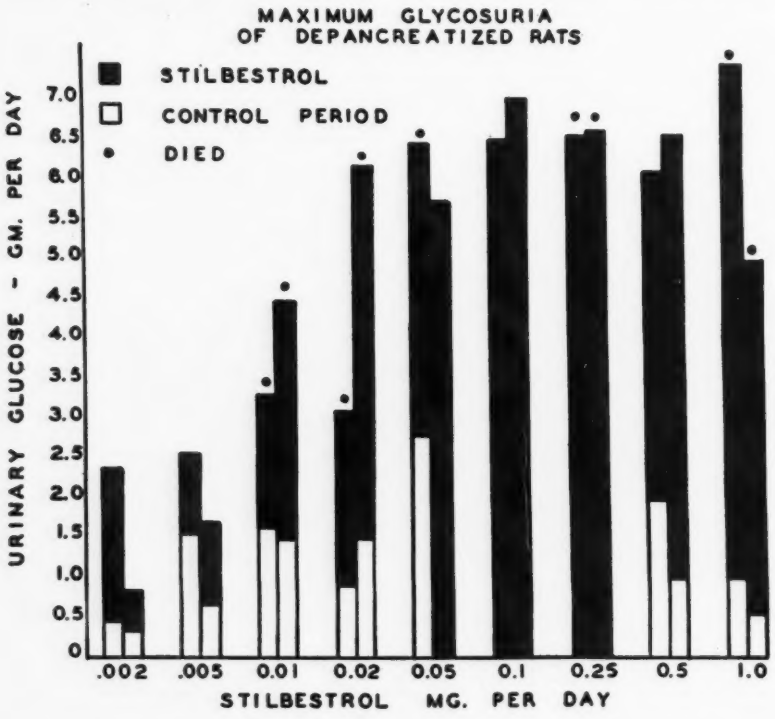


Fig. 5. Maximum values for urinary glucose in 95% depancreatized rats before and during treatment with stilbestrol. Each rat was fed 26 cc. of medium diet daily.

state in one of the normal rats which showed a greater than average response to stilbestrol.

Experiment 2 was an attempt to maintain the state of stilbestrol diabetes in normal rats by increasing the dosage at weekly intervals. Figure 4 gives the values for daily urinary glucose and of body-weight for 6 animals so treated. It was not found possible to maintain a diabetic state by increasing the dosage of stilbestrol.

Experiment 3 was a study of the relationship of the dosage of stilbestrol to the glycosuric response of 95% depancreatized rats which were maintained on medium carbohydrate diet. Some of these rats were spontaneously diabetic. At the time of beginning the administration of stilbestrol they had been observed to be either non-glycosuric or to have a steady glycosuria over a minimum period of 10 days. The injections were continued for a minimum period of 10 days except in those animals which died. The maximum values for urinary glucose during the pre-injection period

and during the injection period are shown in relationship to dosage in figure 5. Figure 6 shows the course of the diabetic state in one animal through two periods of stilbestrol administration.

Experiment 4 was concerned with the specificity of the glycosuric response to stilbestrol. Rats which were 75% and 95% depancreatized were maintained on the high carbohydrate diet. The pre-injection period covered a minimum period of 10

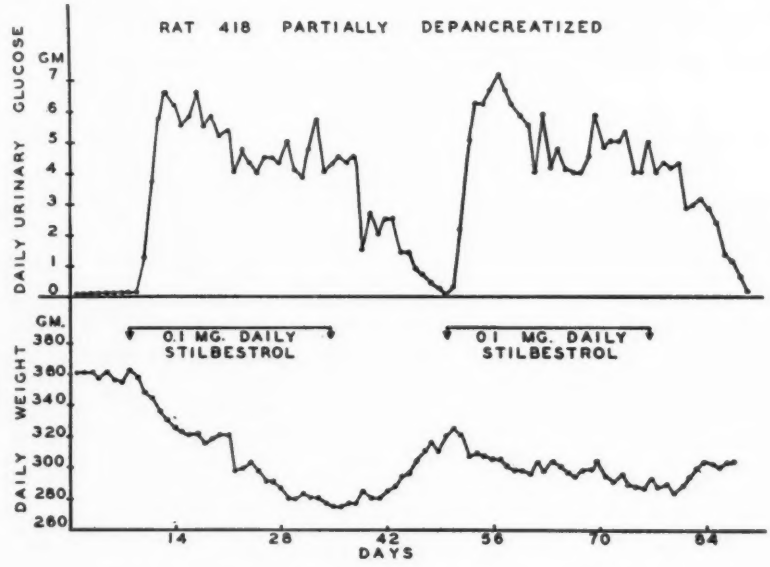


Fig. 6. STILBESTROL DIABETES IN A 95% DEPANCREATIZED RAT FED MEDIUM DIET.

days of non-glycosuria or a steady diabetic state. In addition to estrogenic and androgenic test substances certain non-specific damaging agents were used. The data is presented in table 1.

Urinary glucose. As shown in figures 2, 3 and 4 the diabetic state induced by stilbestrol in normal rats was mild and of short duration. The range of the periods of duration was 2 to 36 days. Even when the dosage was increased, adaptation occurred (fig. 4) so that the glycosuria disappeared. All of the 75% and all of the 95% depancreatized rats responded to stilbestrol by an increased excretion of glucose (fig. 5, 6 and 7). Some of these animals died early during treatment. A number of them became totally diabetic during treatment. There was some evidence of adaptation so that the glycosuria decreased in severity as treatment was continued. Four rats (fig. 6, *rat 418* is typical) were non-glycosuric during the first control period, became severely diabetic during the 28-day period of injection, non-glycosuric during the second control period, severely diabetic during the second 28-day period of injection, and non-glycosuric during the third and final control period. As shown in table 1, dihydrostilbestrol, estradiol, and equilin were also effective in increasing the severity of the diabetic state of partially depancreatized rats. When testosterone and methyl testosterone were administered in massive doses to 95% depancreatized rats they were effective in increasing the severity of the diabetic state. These two androgens

TABLE 1. EFFECT OF ESTROGENS AND ANDROGENS AND OF CERTAIN DAMAGING AGENTS ON GLYCOSURIA OF FORCE-FED PARTIALLY DEPANCREATIZED RATS. All of the rats were maintained on the high carbohydrate diet.

Treatment	Amount per day	Days injected	Rat no.	Maximum glycosuria gm./day		
				Before injection	During injection	After injection
		A. 95% *Pancreas Removed*				
Dihydrostilbestrol, mg.	0.1	14	620	0.00	3.00	0.00
Estradiol, mg.	0.1	7	222	2.50	4.00	2.17
	0.25	4	122	2.30	5.50[1]	
	0.50	10	610	0.00	4.00[1]	
Testosterone, mg.	0.50	14	127	0.00	0.00	0.00
	1.00	14	330	0.00	0.00	0.00
	2.00	10	711	2.56	5.00[1]	
	5.00	14	707	0.00	2.00	0.00
	5.00	14	324	0.00	0.63	0.00
	5.00	10	509	1.65	4.00	1.55
Methyl Testosterone, mg.	0.50	14	228	0.00	0.00	0.00
	1.00	14	620	0.00	0.00	0.00
	2.00	10	608	2.50	2.60	2.80
	2.00	10	707	1.65	1.25	0.94
	5.00	12	618	1.55	5.80[1]	
	5.00	10	707	0.94	3.50	0.64
4% Formaldehyde, cc.	0.2	14	216	2.60	1.65	2.80
	0.5	14	219	1.20	1.60	2.90
	1.0	2	316	2.00	1.75[1]	
2% Formaldehyde, cc.	0.2	14	118	1.00	0.00	0.50
	1.0	7	113	2.77	0.00[1]	
50% Carbon-tetrachloride, cc.	0.5	14	22	0.66	0.60	1.32
	0.5	14	527	0.00	0.00	0.00
Water, cc.	40.0	6	117	1.25	1.11[1]	
Cold-room	24 hours	7	322	0.71	0.00[1]	
		B. 75% *Pancreas Removed*				
Dihydrostilbestrol, mg.	0.50	14	612	0.00	2.80	0.00
	0.50	14	518	0.00	1.70	0.00
Equilin, mg.	0.10	10	701	0.00	0.00	0.00
	0.10	10	702	0.00	0.00	0.00
	0.25	10	703	0.00	2.80	0.00
	0.25	10	812	0.00	2.22	0.00
	0.50	10	719	0.00	2.00	0.00
Testosterone, mg.	2.00	14	703	0.00	0.00	0.00
	5.00	14	726	0.00	0.25	0.00
	5.00	14	612	0.00	0.00	0.00
Methyl Testosterone, mg.	5.00	14	613	0.00	0.00	0.00
	5.00	14	728	0.00	0.00	0.00

[1] Died.

were tested in doses smaller than 1.0 mg. daily with negative results. On the basis of preliminary experiments, doses of formaldehyde, carbon tetrachloride and water were selected which caused damage to the test animals and symptoms of shock. Exposure of the partially depancreatized rat to these damaging agents and to cold

(table 1) failed to increase the severity of the diabetic state. On the contrary there appeared to be a tendency for the glycosuria to decrease during the first few days that the animal was subjected to the damaging agent. This point was not clearly established.

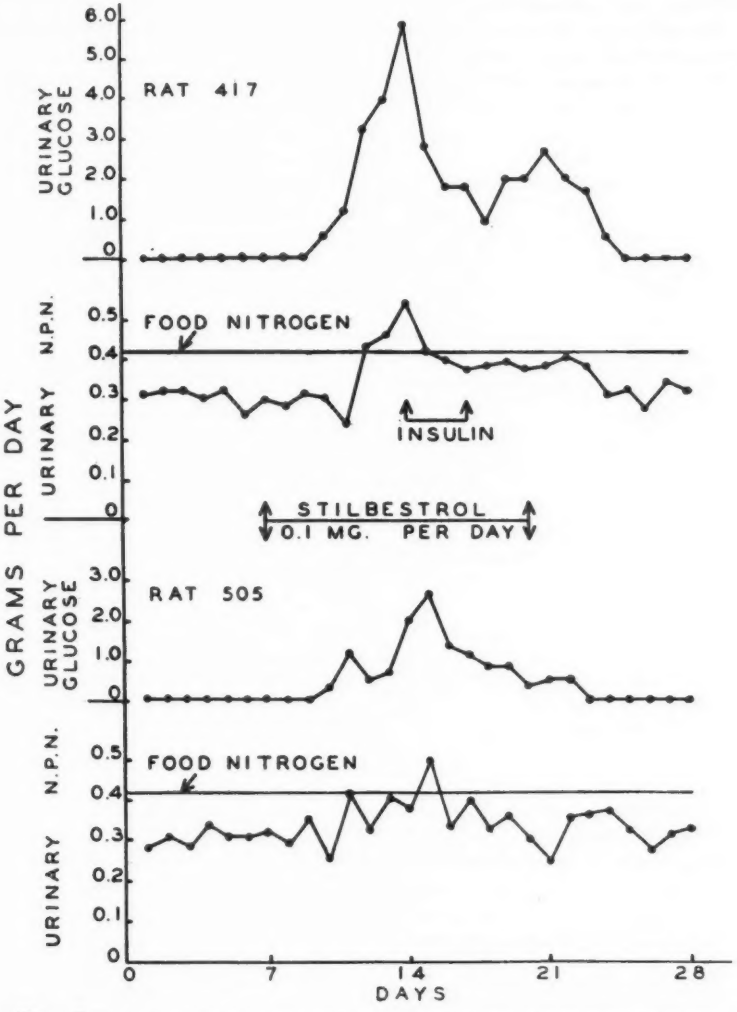


Fig. 7. Effect of stilbestrol on excretion of glucose and non-protein nitrogen in two 75% depancreatized rats maintained on high carbohydrate diet.

Body-weight. All of the normal rats gained in weight during the control period and during the administration of stilbestrol except when they were excreting glucose. The partially depancreatized rats gained in weight during the control periods but lost weight during the periods of glycosuria. Those animals which died during treat-

ment showed a sharp loss in weight of 25 to 40 gm. during a period of a few hours prior to death.

Urinary ketones. All of the normal and 75% depancreatized rats were free of ketonuria during all phases of the experiments. Several of the 95% depancreatized rats which were maintained on the medium diet showed ketonuria on days when all the available carbohydrate of the diet was excreted. None of the 95% depancreatized animals excreted ketones while fed the high carbohydrate diet.

Urinary non-protein nitrogen. The level of urinary non-protein nitrogen was studied in 4 normal rats, 4 rats which were 75% depancreatized, and 4 rats which were 95% depancreatized. In each animal the excretion of nitrogen was less than that contained in the food during the pre-injection period and was uniformly increased toward or above the level of food nitrogen during treatment with stilbestrol. The

TABLE 2. BLOOD SUGAR VALUES IN RATS BEFORE AND DURING THE ADMINISTRATION OF STILBESTROL. The normal and 75% depancreatized rats were fed the high carbohydrate diet and the 95% depancreatized rats were fed the medium diet. *Rat 222* had a spontaneous glycosuria during the control period. All of the other rats were non-glycosuric during the control period.

Rat no.	Experimental condition	Phase of experiment	Blood sugar (mg. %)				
			16 hour fast	Hours following feeding			
				2	4	6	8
2	Normal	Control	85	116	119	90	80
		Injection	88	298	322	265	224
208	Normal	Control	86	122	128	100	85
		Injection	98	278	310	284	218
417	75% depan.	Control	85	120	138	134	109
		Injection	103	322	352	358	345
505	75% depan.	Control	90	115	122	92	84
		Injection	108	300	328	330	326
418	95% depan.	Control	105	129	135	142	125
			250	342	355	348	348
222	95% depan.	Control	115	220	275	260	190
			265	338	349	345	350

effect of stilbestrol on nitrogen excretion is illustrated in figure 7, on two 75% depancreatized animals. However, only a small part of the increased glucose excretion of these animals could be accounted for by the increase in formation of glucose from protein on the basis of a glucose:nitrogen ratio of 3.6.

Blood-sugar. Observations on the levels of blood sugar before and during the administration of stilbestrol showed clearly that stilbestrol diabetes is characterized by hyperglycemia. Data on 6 representative animals is presented in table 2.

Pathology. Only superficial observations were made on the pathologic changes occurring in the animals. Those animals which were treated with stilbestrol and other estrogens showed a marked hypertrophy of the adrenal cortices and extensive atrophy of the thymus and testes. A bloody appearing discharge from the eyes and nose was common. Diarrhea was seldom observed, and lesions in the intestinal tract were observed only in those animals which died. All of the animals remained vigorous and active until they became severely diabetic. All of the rats treated with estrogens exhibited a lordosis response when stimulated by pressure about the genitalia. The

cause of death among rats made diabetic with estrogens is not clearly understood. Death was always preceded by rapid dehydration due to failure to drink water at a time when large amounts of water were being excreted. Death was found to occur in the absence of acidosis. The syndrome exhibited by these animals was identical with that shown by either normal or partially depancreatized rats when given a full feeding of high carbohydrate diet by stomach tube without an adaptation period. In all cases death occurred during a rapid increase of glycosuria with an accompanying polyuria. Deaths could be uniformly prevented by early treatment with insulin or delayed by the forcing of fluids. It seems probable that dehydration was a major cause of death of these animals but there are many points concerning the syndrome which are not clear.

Those rats which were treated with androgens in diabetogenic doses showed hypertrophy of the adrenal cortices and atrophy of thymus and testes. The rats treated with formaldehyde showed massive edema at the site of injection so that these animals gained 100 gm. or more by fluid retention within 3 or 4 days. These animals showed a fall in temperature and became weak. At necropsy the adrenal cortices were found to be enlarged and the thymus atrophic. At necropsy the two rats treated with carbon tetrachloride showed extensive liver cirrhosis, adrenal cortical hypertrophy and thymus atrophy. The rat which was given the intraperitoneal injections of water showed a fall in body temperature, weakness, and hematuria. At necropsy the adrenal cortices were enlarged and the thymus atrophic. Similar changes, without hematuria, occurred in the rat maintained at 0°C. for one week.

DISCUSSION

Very small doses of stilbestrol were effective in increasing the severity of the diabetic state of partially depancreatized rats. Positive effects were obtained with as little as 2γ daily and 10γ daily was as effective as any larger dose. In the normal rats the smallest effective dose was 50γ daily and larger amounts up to 500γ daily produced greater glycosuric responses. Since only a small number of animals were tested at each dose level these values are not reliable in a statistical sense, but they do illustrate the fact that small amounts of the compound are diabetogenic. Since stilbestrol is estrogenic in amounts as small as 0.5γ per day it is apparent that it is a highly active compound from both the physiologic and pharmacologic standpoint. The natural estrogens share the diabetogenic property of stilbestrol. No effort was made in this study to compare the diabetogenic activity of different estrogens quantitatively. However, it is apparent from table 1 that the four estrogens studied manifest diabetogenic activity in smaller doses than do either testosterone or methyl testosterone, Dolin, Joseph and Gaunt (3) reported some slight diabetogenic effect of testosterone in the depancreatized ferret.

Superficially at least, the diabetogenic effect of estrogens is like that of the adrenal steroids. In each case the diabetogenic state is characterized by hyperglycemia and an increased excretion of non-protein nitrogen which is not sufficiently great to account for the extra glucose excreted (5, 6). In each case it is necessary to assume that some part of the carbohydrate of the food is being excreted due to altered utilization or interconversion. The fact that estrogens cause great hypertrophy of the adrenal cortices gives a basis for assuming that stimulation of the secretory activity of these organs may mediate the diabetogenic activity of the estrogens. But, the administration of non-specific damaging agents (*experiment 4*, table 1) which also cause hypertrophy of the adrenal cortices failed to have any similar effect on the diabetic state. Estrogens are also known to cause enlargement of the anterior pituitary (7).

Further studies are needed to elucidate the possible mediating rôle of these other endocrine organs.

As shown in figure 4, rats continue to gain in weight when treated with stilbestrol if they are force-fed. This finding is consistent with the conclusions of Nelson (8) that the commonly observed weight reducing action of estrogens is due to depression of appetite.

Dolin, Joseph and Gaunt (3) have pointed out that there have been reported two types of effects of estrogens on carbohydrate metabolism, those in which the blood sugar or liver glycogen is elevated (9–13) and those in which the severity of the diabetic state is decreased (14–20). At present there is no satisfactory explanation of how a substance may at one time be diabetogenic and at another time decrease the severity of diabetes. The relative importance of species differences and of differences in the experimental conditions must be studied further before this paradox can be evaluated. In recent studies Young (21) found that the administration of estrogens to dogs made permanently diabetic with pituitary extracts failed to modify the diabetic state and Lawrence and Madders (22) reported a lack of effect of stilbestrol on the diabetes of patients.

SUMMARY

The administration of stilbestrol to partially depancreatized non-glycosuric or mildly glycosuric rats on a constant medium carbohydrate diet produced a severe glycosuria and hyperglycemia in all cases. Similar results were obtained with dihydrostilbestrol, estradiol and equilin. Massive doses of testosterone and methyl testosterone were weakly diabetogenic. The administration of stilbestrol to normal non-glycosuric rats on a constant high carbohydrate diet produced a mild temporary glycosuria in the majority of cases.

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REFERENCES

1. Ingle, D. J.: *Am. J. Med. Sci.* 201: 153. 1941.
2. Ingle, D. J.: *Am. J. Physiol.* 133: P337. 1941.
3. Dolin, D., S. Joseph and R. Gaunt: *Endocrinology* 28: 840. 1941.
4. Evans, G.: *Am. J. Physiol.* 114: 297. 1936.
5. Ingle, D. J., and G. W. Thorn: *Am. J. Physiol.* 132: 670. 1941.
6. Ingle, D. J.: *Endocrinology.* In Press.
7. Nelson, W. O.: *Am. J. Physiol.* 133: P398. 1941.
8. Nelson, W. O.: Personal communication.
9. Gulick, M., L. T. Samuels and H. J. Deuel, Jr.: *J. Biol. Chem.* 105: 29. 1934.
10. Janes, R. G., and W. O. Nelson: *Proc. Soc. Exper. Biol. & Med.* 43: 340. 1940.
11. Zunz, E., and J. LaBarre: *Arch. Internat. Physiol.* 48: 287. 1939.
12. Collazo, J. A., and B. Marti: *Ann. de med.* 38: 383. 1935.
13. Griffiths, M., H. P. Marks and F. C. Young: *Nature.* London, 147: 359. 1941.
14. Barnes, B. O., J. F. Regan and W. O. Nelson: *J.A.M.A.* 101: 926. 1933.
15. Nelson, W. O., and M. D. Overholser: *Endocrinology* 20: 473. 1936.
16. Mazer, C., D. R. Meranze and S. L. Israel: *J.A.M.A.* 105: 257. 1935.
17. Gessler, C. J., J. A. Halsted and R. P. Stetson: *J. Clin. Invest.* 18: 715. 1939.
18. Spiegelman, A. R.: *Am. J. Med. Sci.* 200: 228. 1940.
19. Bartelheimer, H.: *Dtsch. Arch. f. klin. Med.* 184: 185. 1939.
20. Cantilo, E.: *Endocrinology* 28: 20. 1941.
21. Young, F. G.: *The Lancet* 240: 600. 1941.
22. Lawrence, R. D., and K. Madders: *The Lancet* 240: 601. 1941.

INSULIN CONCENTRATION IN THE BLOOD OF NORMAL AND PANCREATECTOMIZED DOGS[1]

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IT WAS PREVIOUSLY FOUND (1) that insulin can be detected in human blood by injecting it intraperitoneally into the hypophysectomized-adreno-demedullated rat. If 1 cc. of human blood was injected per 100 gm. of rat the blood sugar fell from 58 to 51 mg.% indicating an insulin content of 0.0002 U of insulin per cc. In order to determine the specificity of this reaction for insulin, experiments were carried out on pancreatectomized dogs. They were supplemented by studies on the influence of insulin injected into these animals on the insulin content of the blood.

METHOD AND RESULTS

The blood to be tested for insulin was injected immediately into the peritoneal cavity of the hypophysectomized-adreno-demedullated rat. The procedure was the

TABLE 1. INSULIN CONTENT[1] OF DOG'S BLOOD

Dog No.	Blood sugar	Test		Insulin
		A	B	
	mg. %	*mg. %*	*mg. %*	U/cc.
1	75	59	47	0.00009
2	85	59	47	
3	73	60	46	
4	97	60	49	0.00008
5	108	60	49	
6	74	59	47	0.00009
7	81	60	42	0.00012
8	81	58	45	0.0001

[1] 4 cc. of blood per 100 gm. rat are injected into the hypophsectomized-adreno-demedullated rat. The blood sugar of the test animal before the injection is listed as *A*, the blood sugar of the rat 1 hour after the injection, as *B*. The insulin content in units is derived from the insulin assay graphs published in an earlier investigation.

same as reported previously, except that 4 cc. of blood per 100 gm. rat were injected. The blood was tolerated by the animals without any ill effects.

Table 1 shows the hypoglycemic reaction produced by the injection of normal dog's blood into the hypophysectomized-adreno-demedullated rat. It was found that the blood sugar in the test animal was lowered from 60 to 48 mg.%, which corresponds to approximately 0.0001 U of insulin per cc. of blood. Apparently the insulin content of dog's blood tested 24 hours after feeding is practically identical with that of the human blood.[2] Several tests performed with rabbit's blood under similar conditions gave practically identical values.

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[1] Aided by a grant from the John and Mary R. Markle Foundation. Assistance was also given by the W.P.A. Project, Ill. #30278.

[2] If human blood was assayed by injecting 4 cc. of blood/100 gm. rat the blood sugar of the test animal fell to 44 mg.% indicating 0.0001 U of insulin per cc. of blood.

That the hypoglycemic reaction resulting from the injection of blood is due to insulin is made probable by the observation that the injection of blood does not alter the blood sugar of the rat if this animal is not greatly sensitized to insulin. We have found invariably that if the hypophysectomy was incomplete, no fall in blood sugar was observed in the test animal. But the assay of the blood of pancreatectomized animals is a more specific test to determine whether the hypoglycemic reaction produced by normal blood is due to insulin.

From the following protocols, it is obvious that pancreatectomized dogs which had not been given insulin for 48 hours did not have any insulin in the blood when tested on the hypophysectomized-adreno-demedullated rat. In *dog 1* the injection of blood resulted in a change of the blood sugar of the test animal from 59 to 57 mg.% when the blood sugar of the animal was 230 mg.% (*test A*). In a second test (*E*) performed 10 days later the injection of the blood did not alter the blood sugar at all and the blood sugar level of the pancreatectomized dog was 335 mg.%.

In *dog 2* two tests (*B* and *G*) were performed on 4/14/41 and 4/24/41. They did not produce any blood sugar change in the test animal at a time when the blood sugar of the pancreatectomized dog was 258 and 173 mg.% respectively. If, however, insulin was injected, thus reducing the diabetic blood sugar level, the assay of the blood in the hypophysectomized-adreno-demedullated rat showed insulin was present.

Protocols

Dog. 1. Male; 8 kg. Pancreatectomy, 3/21/41; 3/22/41, 4 U protamine-insulin intramuscularly; 3/24/41, blood sugar 232 mg.%; *A test*,[1] 59(A)–57(*B*) mg.%. 6 U protamine-insulin given daily from 3/24/41 to 3/28/41; 3/28/41, 3:00 P.M., blood sugar 90 mg.%; *B test*, 60(A)–46(*B*) mg.%. 3/29/41, 12:30 P.M., blood sugar 167 mg.%; *C test*, 59(A)–52(*B*) mg.%; 3/29/41, 12:40 P.M., 6 U protamine-insulin intramuscularly; 3/31/41, 9:00 A.M., 6 U protamine-insulin intramuscularly; 3/31/41, 12:00 P.M., 10 U insulin[2] intramuscularly; 3/31/41, 1:00 P.M., blood sugar 76 mg.%; 3/31/41, 1:43 P.M., 4 U insulin[2] intravenously; 3/31/41, 2:18 P.M., blood sugar 51 mg.%; *D test*, 60(A)–26(*B*) mg.%. 4/1/41, 9:00 A.M., 6 U protamine-insulin intramuscularly; 4/3/41, 2:30 P.M., blood sugar 335 mg.%; *E test*, 58(A)–58(*B*) mg.%. 4/3/41, 2:35 P.M., 6 U protamine-insulin; 4/4/41, 9:00 A.M., 6 U protamine-insulin; 4/4/41, 12:00 M., 16 U insulin[2] intramuscularly; 4/4/41, 2:10 P.M., blood sugar 65 mg.%; 4/4/41, 2:45 P.M., blood sugar 57 mg.%; *F test*, 61(A)–35(*B*) mg.%. 4/4/41, 2:50 P.M., 15 cc. 15% glucose intravenously; 4/4/41, 2:51 P.M., blood sugar 122 mg.%; *G test*, 58(A)–34(*B*) mg.%.

Dog 2. Female; 12 kg. 4/9/41, blood sugar 73 mg.%; *A test*, 60(A)–46(*B*) mg.%. 4/10/41, total pancreatectomy; 4/11/41, 9:45 A.M., 8 U protamine-insulin intramuscularly; 4/12/41, 12:00 M., 8 U protamine-insulin intramuscularly; 4/14/41, 1:20 P.M., blood sugar 258 mg.%; *B test*, 59(A)–60(*B*) mg.%. 4/14/41, 1:23 P.M., 2 U per kg. of insulin[2] intramuscularly; 4/14/41, 2:23 P.M., blood sugar 85 mg.%; *C test*, 58(A)–44(*B*) mg.%. 4/14/41, 3:25 P.M., blood sugar 49 mg.%; *D test*, 61(A)–23[3](*B*) mg.%. 4/14/41, 3:30 P.M., 8 U protamine-insulin; 4/15/41, 1:30 P.M., blood sugar 79 mg.%; *E test*, 59(A)–44(*B*) mg.%. 4/15/41, 2:10 P.M., 2 U per kg. insulin[2] intramuscularly; 4/15/41, 3:10 P.M., blood sugar 55 mg.%; *F test*, 60(A)–34(*B*) mg.%. Daily injection of 8 U protamine-insulin up to 4/19/41; 4/21/41, 2:34 P.M., blood sugar 173 mg.%; *G test*, 58(A)–57(*B*) mg.%. 4/21/41, 2:35 P.M., 2 U insulin[2] intramuscularly; 4/21/41, 3:05 P.M., blood sugar 91 mg.%; *H test*, 62(A)–50(*B*) mg.%.

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[1] 4 cc. of blood per 100 gm. injected intraperitoneally into the hypophysectomized-adreno-demedullated rat. The first value (*A*) shows the blood sugar of the test animal before, the second (*B*) one hour after the injection of blood.

[2] Eli Lilly and Company, Indianapolis, Ind.

[3] In this case hypoglycemic convulsions started after 30 minutes. Consequently the (B) sample was obtained at that time and not as usually after one hour.

Several observations reported in the protocols deserve comment. It was found that, by the injection of insulin, the blood sugar of the pancreatectomized dog was reduced to the normal level when the assay of the blood for insulin showed that the insulin concentration of the blood was identical with that in normal animals (cf. *test B* in *dog 1*; and *test C*, *E* and *H* in *dog 2*.) If, however, less insulin was injected than is necessary to lower the blood sugar to the normal value, the assay showed the insulin content of the blood to be less than normal. On the other hand, the injection of excessive amounts of insulin, lowering the blood sugar below the normal value, showed insulin amounts in the blood which were greater than normal when tested with the hypophysectomized-adreno-demedullated rat (*test D* and *F* in *dog 1* and *2*).

Since the assay of insulin was performed on blood samples with widely varying blood sugar concentration it is important to determine whether or not the blood sugar has a significant influence on the assay which is independent of the insulin content. In order to test this question a glucose solution was injected into the pancreatectomized dog and immediately before and after the injection of glucose the blood was tested for insulin. The protocol on *dog 1* (4/4/41, 2:45 P.M.) gives a typical example. When the blood sugar of the dog was 57 mg.% the injection of this blood into the test animal lowered the blood sugar to 35 mg.%. If a glucose solution was injected, raising the blood sugar to 122 mg.%, the immediate injection of this blood into the test animal gave almost exactly the same hypoglycemic reaction (the blood sugar of the test animal fell to 34 mg.%) as had been observed before glucose was injected. Similar observations were made in man and in normal dogs. Thus, in one experiment the blood sugar of a dog was raised from 85 mg.% to 178 mg.% by intravenous injection of 0.5 gm. glucose/kg. The blood was assayed before and after the injection of glucose. In spite of the great difference in the blood sugar level, the tests did not show any significant changes. The blood sugar of the test animal fell from 59 to 47 mg.% before and from 58 to 48 mg.% immediately after the injection.

The experiments show clearly that the insulin assay is not determined by the amount of sugar present in the blood, but by its insulin concentration. The complete absence of any hypoglycemic effect in the test animals when blood of a pancreatectomized dog was injected indicates that there is no other source of insulin in the blood if the pancreas is removed. These observations agree with the work of Best, Jephcott, and Scott (2) who failed to find insulin in tissues other than the pancreas.

SUMMARY

The insulin concentration of dog's blood is determined by injecting 4 cc. of blood intraperitoneally into the hypophysectomized-adreno-demedullated rat and determining the blood sugar in the test animal one hour after injection. It is found that the normal blood of dogs fasted for 24 hours contains approximately 0.0001 U of insulin per cc. The hypoglycemic reaction of the test animal to dog's blood is completely abolished after pancreatectomy of the latter. This therefore proves the specificity of the test for insulin in the blood. If insulin in various quantities is injected in the pancreatectomized dog and the blood is then tested on the hypophysectomized-adreno-demedullated rat for insulin it is found that insulin concentration and blood sugar are inversely related. Control experiments show that the alternation of the blood sugar itself induced by the injection of glucose does not alter the insulin concentration in the blood. The only source of insulin which appears in the blood is the pancreas.

REFERENCES

1. Gellhorn, E., J. Feldman and A. Allen: *Endocrinology* 29: 137. 1941.
2. Best, C. H., C. M. Jephcott and D. A. Scott: *American J. Physiol.* 100: 285. 1932.

NOTES AND COMMENTS

BODY AND MUSCLE GROWTH IN NORMAL AND CASTRATE ALBINO MICE

IT IS WELL KNOWN that castrate men and animals grow taller and fatter than the normal male. Generally it is accepted that the epiphyses are closed later. Rubinstein and Solomon[1] recently reported more rapid growth in rats treated with small quantities of testosterone than in untreated castrated animals. Other authors, too, have observed that castrated rats remain shorter than normal.[2–7] This is contrary to the observations made on horses and cattle. Many authors[8–14] have observed that male sex hormones inhibit the growth of animals, probably by their influence on the growth hormone production of the anterior hypophysis. Shay et al.[15] observed that testosterone propionate had no influence on the growth of male mice.

We made some experiments in order to study this question, using white male mice, but we castrated them at a much older age than Rubinstein and Solomon did their rats. In this country castration generally is practiced in horses and cattle which are nearly adult. The adult weight of our mice averages 20 gm. One group of 10 mice was castrated and another group of 10 mice was used as controls. The initial weight of the animals was 16 to 18.5 gm. The results follow.

	Initial wt. gm.	Wt. after 2 mo. gm.	% Wt. gain
Normal ♂	18.3	19.8	8.2
Castrate ♂	16.98	19.4	7.75

In the later growth periods there was almost no difference in weight between normal and castrated male animals. The adult weight of the castrated and the normal animals was the same.

It seemed of some interest to determine if there is a difference in the growth of muscles in the normal and castrate animal. As it would be difficult to detect differences in the study of one muscle in these small animals, the weight of a group of muscles of the leg (together with the tibia) was used. In every animal the tibia with the following muscles was weighed, *quadriceps femoris, m. adductor, m. biceps femoris, m. semimembranosus, m. semitendinosus,*

[1] RUBINSTEIN, H. S., AND M. L. SOLOMON: *Endocrinology* 28: 229. 1941.
[2] DONALDSON, H. H., AND S. HATAI: *J. Comp. Neurol.* 21: 155. 1911.
[3] KORENSCHEWSKY, V.: *J. Pathol. & Bacter.* 33: 607. 1930.
[4] EVANS, H. M., AND M. E. SIMPSON: *Anat. Rec.* 35: 36. 1927.
[5] LAWLESS, J. J.: *Anat. Rec.* 66: 455. 1938.
[6] RUBINSTEIN, H. S., A. K. ABARBANEL AND A. A. KURLAND: *Endocrinology* 25: 397. 1939.
[7] RUBINSTEIN, H. S., A. A. KURLAND AND M. GOODWIN: *Endocrinology* 25: 724. 1939.
[8] STEINACH, E., AND G. HOLZKNECHT: *Arch. f. Entwicklungsmech* 42: 490. 1917.
[9] SPENCER, J., F. E. D'AMOUR AND P. G. GUSTAVSON: *Endocrinology* 16: 647. 1932.
[10] ZONDEK, B.: *Lancet.* 230: 10. 1936.
[11] ZONDEK B.: *Lancet* 231: 842. 1936.
[12] MCCULLAGH, D. R., AND E. L. WALSH: *Endocrinology* 19: 466. 1935.
[13] HAMILTON, S. B., AND J. M. WOLFE: *Endocrinology* 22: 360. 1938.
[14] BUGBEE, E. P., AND A. E. SIMOND: *Endocrinology* 10: 360. 1926.
[15] SHAY, H., J. GERSHON-COHEN, K. PASCHKIS AND S. S. FELS: *Endocrinology* 28: 877. 1941.

m. extensor fasciae latae, m. tibialis anterior, flexor digitorum profundus, flexor digitorum superficialis, m. popliteus, m. gastrocnemius, m. soleus, m. peroneus anterior.

The weight of these muscle groups was 0.09 gm. in the normal males; in the castrate animals 0.078 gm. In the normal males this was 4.63, in the castrate group, 3.94% of body weight. The muscles of the normal males had grown in two months, in relation to the body weight 14.8% heavier than the muscles of the castrate group.

In albino mice there was no difference in the body weight of normal and of castrated animals in the later periods of growth. There was, however, a remarkable difference in the development of a muscle group of the leg in relation to the body weight, the muscle group being much heavier in the normal males.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE ANNUAL MEETING

THE TWENTY-SIXTH ANNUAL MEETING of the Association for the Study of Internal Secretions will be held in Atlantic City, New Jersey, Monday and Tuesday, June 8th and 9th, 1942. Headquarters will be the Hotel Traymore.

Full directions for submission of titles and abstracts of papers for presentation at the Annual Meeting will appear in the next issue of this *Journal*.